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(71) Applicant *(for all designated States except US)*: MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 1B1 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BATIST, Gerald [CA/CA]; 4670 Grosvenor Avenue, Montréal, Québec H3W 2L8 (CA). KATABI, Maha [CA/CA]; 4866 Côte des Neiges #301, Montréal, Québec H3V 1H1 (CA).

(74) Agent: COTE, France; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).

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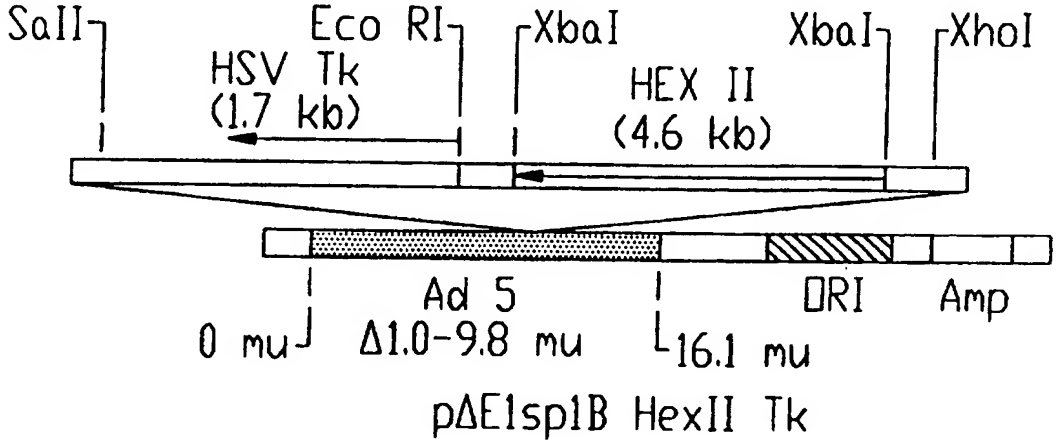
(54) Title: HEX II TUMOR-SPECIFIC PROMOTER AND USES THEREOF IN CANCER THERAPY



(57) Abstract

The present invention relates to a tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II promoter. The present invention also relates to a gene construct, which include Hex II promoter in a vector selected from pCAT basic expression vector pΔE1sp1B and a shuttle plasmid, and which optionally includes β-gal or HSV Tk.

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HEX II TUMOR-SPECIFIC PROMOTER AND USES THEREOF IN CANCER THERAPY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, such as to drive a suicide gene in cancer therapy.

(b) Description of Prior Art

A successful gene therapy approach is dependent upon two parameters: 1) efficiency of target cells transduction and 2) specificity of gene delivery. Selective targeting is especially critical in the context of cancer therapy for gene directed enzyme prodrug therapy (GDEPT), where a suicide gene expressed in tumor cells encodes an enzyme that converts an otherwise non-toxic prodrug into its active form.

Several methods have been explored to increase the specificity. They can be broadly divided into two categories: directed delivery of the gene of interest or its directed expression. The ideal candidate for transcriptional targeting would be a tumor specific promoter and/or enhancer and its activation will be strong enough to achieve therapeutic levels of the desired transcript. A wide range of promoters have been explored in this context. They were mostly characterized as tissue specific promoters as opposed to tumor selective. Some examples are: surfactant protein SP-A promoter for non small cell lung carcinoma (NSCLC), immunoglobulin enhancer or O enhancer for B-cell lineage cancers, tyrosinase for melanomas, and MUC-1/Df3 for breast cancer. However, these promoters also direct gene expression in the normal tissue of origin of these neoplasms and other critical organs as

well. The erbB2 and a-fetoprotein promoters are activated to a greater extent in certain neoplasms. They have also been used in this strategy and have lead to promising results. Nonetheless, other promoters to further improve and optimize this strategy are needed.

A striking characteristic of rapidly growing tumor cells is their high rate of glucose utilization compared to their normal counterparts. Glucose is mainly channeled through the glycolytic pathway which is not only used for rapid energy production but also for the provision of biosynthetic precursors necessary to sustain a high rate of cellular division. Hexokinase (ATP: D-hexose-6-phosphotransferase) catalyses the first committed step of glycolysis; therefore it was suspected by many to be a potential player in this phenotype. Hexokinases (HK) are comprised of two highly homologous 50kDa halves and are product inhibited by glucose-6-phosphate to varying degrees. They exist in four molecular forms, HK I to HK IV, with distinct electrophoretic and kinetic properties (Wilson, J.E., (1985) *In Regulation of Carbohydrate Metabolism*, Vol I, 45-85, CRC Press, Boca Raton). The profile of these enzymes in tissues at different stages of malignancies shows an increase in HK II in tumor versus normal tissues. In rats, the type I HK is expressed in brain, kidney and heart. The type II HK was found in skeletal muscle and in AH130 hepatoma cells. In normal liver it is type IV HK that is most abundant (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

Comparison of the rat hexokinase II with a hexokinase from rat Novikoff ascites shows there is a single type II isozyme that is found in both normal and tumor tissues (Adams, V., Kempf, W, Hassam, S., and Briner, J. (1995) *Biochem. Mol. Med.* **54**, 53-58). The

inhibition of HK II by glucose-6-phosphate is delayed. Therefore, tumors are able to build up high levels of this product. Its accumulation is a signal for glucose availability for consumption, a stimulus of biosynthetic pathways for growth (Wilson, J.E., (1985) *In Regulation of Carbohydrate Metabolism*, Vol I, 45-85, CRC Press, Boca Raton). The level of HK II was also found to be increased in human HepG2 cells and in renal cell carcinoma (Adams, V., Kempf, W, Hassam, S., and Briner, J. (1995) *Biochem. Mol. Med.* **54**, 53-58). Two factors are involved in this increased activity: the propensity of the tumor enzyme to bind to the outer mitochondrial membrane (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925) and overproduction of the enzyme. The latter is due to both a gene amplification of the tumor type II isozyme and to its transcriptional upregulation (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925). The promoter for the rat tumor type II enzyme has recently been cloned. Regulation of the promoter with known modulators of glucose metabolism was found to be different in hepatoma cells and normal rat hepatocytes (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

It would be highly desirable to be provided with a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells compared to normal cells.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer

cells, such as to drive a suicide gene in cancer therapy.

In accordance with the present invention there is provided a tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II reporter gene.

In accordance with the present invention there is also provided a Hex II gene construct, which comprises Hex II promoter in a vector selected from pCAT basic expression vector pΔE1splB and a shuttle plasmid.

In accordance with one embodiment of the present invention the gene construct further comprises β-gal or HSV Tk.

In accordance with another embodiment of the present invention, the preferred gene construct based on pCAT vector is pHexII4557-CAT.

In accordance with another embodiment of the present invention, the preferred gene constructs based on pΔE1splB are pΔE1splBHex-LacZ and pΔE1splBHex-TK.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the Hex II reporter gene construct in pCAT basic expression vector in accordance with the present invention;

Fig. 2 illustrates the Hex II promoter construct including β-galactosidase in the shuttle plasmid pΔ E1splB in accordance with the present invention;

Fig. 3 illustrates the Hex II promoter construct including HSV Tk in the shuttle plasmid pΔE1splB in accordance with the present invention;

Fig. 4 illustrates a graph of the results of MUC-1 versus HexII promoters activation in normal bronchial and mammary epithelial cells; and

Fig. 5 illustrates a graph of the results of HexII promoter activation in normal bronchial epithelial cells versus non-small cell lung carcinomas.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a new HexII promoter. Its constructs are illustrated in Figs. 1 to 3.

1. Construction of recombinant plasmids

pHexII4557-CAT

(8.9 kb) The HexII, 5.15 kb, promoter in the plasmid pUC18 (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925) was released with an Xbal digest and cloned into the pCAT basic vector (Promega). The size of the promoter was reduced to 4.56 kb with a BamHI digest that released sequences from the non coding region at the 3' end of the clone.

pΔElsplBHex-LacZ

(14.7 kb) the 3.74 kb lacZ gene (HindIII-SalI) from pSV2-β-galactosidase was cloned into the HindIII and SalI polycloning sites of the shuttle vector pΔ ElsplB. This shuttle plasmid contains Adenovirus 5 (Ad5) sequences from map unit 0 to 1, followed by the polycloning site, followed by Ad5 sequences from mu 9.8 to 15.8, and therefore allows recombination to take place with the adenoviral genome. The Hex II promoter 4557 bp was released from the pHexII 4557/CAT with XbaI followed by an EcoRI digest and cloned into the XbaI site of the pΔElsplB. Clone 10 (pΔElsplBHexII) that had the insert in the negative orientation relative to the polycloning site of the pΔElsplB was used for further cloning of the Hex LacZ plasmid. pΔElsplBLacZ was digested with XhoI followed with a partial digest with

EcoRI. pΔElsplBHexII was in turn digested with XhoI and EcoRI, and the purified 4.6 kb fragment was ligated into pΔElsplBLacZ.

pΔElsplBHex-TK

(12.6 kb) The 1.7 kb HSV-TK gene (EcoRI-SalI) from pMClTK was cloned into the corresponding sites of pΔElsplB. Subsequently, the resulting pΔElsplBTK plasmid was cut with EcoRI and XhoI, and the purified 4.6 kb HexII fragment with compatible ends was ligated into it. Plasmid DNA was purified by alkaline lysis followed by cesium chloride density gradient purification.

The use of tissue or tumor selective promoters in targeted gene therapy for cancer depends on strong promoters with specific activity. The Muc-1/Df3 promoter has been used in the context of gene directed enzyme prodrug therapy (GDEPT) (Chen et al (1995) *J. Clin. Invest.* **96**(6), 2775). However we have found that it has limited promoter activity and appears to be expressed in a wide range of normal cells (Fig. 4). An interesting property of cancer cells that could be exploited to target them selectively is their increased rate of glycolysis. Hexokinase type II (Hex II) catalyzes the first committed step of glycolysis and has been linked to this phenotype since it is overexpressed in tumors and is not responsive to the normal physiological inhibitors, e.g. glucagon (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

In accordance with the present invention, the tumor HK II promoter was tested in variety of human tumor cell lines and in normal human cells. We studied the Hex II promoter by transfecting cells with the pHex II4557/CAT (Fig. 1) construct and performing a

chloramphenicol acetyl transferase (CAT) reporter gene assay.

2. Transfection and reporter gene assays

Transient transfections were performed using lipofectamine according to the manufacturer's recommendations (GIBCO-BRL). Cells were plated the day before transfection to give 60% confluency in 6-well plates. The pl583/+33MUC1.CAT or pHex4557.CAT vectors were transfected along with pSV_2lacZ to determine promoter activity. 1 ug of each plasmid were used for each well. All conditions assayed were done in duplicate. The plasmids pRSV.CAT and promoterless pCAT were used as positive and negative controls, respectively. Cells extracts were prepared 48 hours after transfection and β-galactosidase activity was assayed to compensate for variations in transfection efficiency. CAT activity was determined from 75-100 ug of proteins. The reaction was carried out with 0.1 uCi of ^{14}C-labeled chloramphenicol in a 100 ul reaction at 37°C for 4 hrs.

Results

Its activation was very high in tumor as opposed to normal cells. The activation of HeX II in the non-small cell lung carcinomas H661 and H460 was 43% and 64% (respectively) of the activation observed with the Rous Sarcoma virus (RSV) constitutive promoter while it was 3% of RSV in the primary normal human bronchial epithelial cells (NHBEC). Moreover, treatment of the transfectants with glucagon did non inhibit promoter activation in H661 cells. Its activation in the human mammary carcinoma cells MCF-7 was 72% of RSV while it was 23% of RSV in the normal human mammary epithelial cells (NHMEC).

Moreover, the efficacy of this promoter in the context of GDEPT was tested by using the herpes thymidine kinase gene in combination with the prodrug gancyclovir.

The following suicide genes may be used in accordance with the Hex II promoter constructs of the present invention: Cytochrome P-450™ 2B1 with cyclophosphamide, penicillin, amidase and β-lactamase.

3. MTT cell viability assays

Cell survival was determined using a colorimetric assay which measures the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to an insoluble purple formazan precipitate. Cells in the logarithmic phase of growth were resuspended at a concentration of $2x10^5$ cells/ml. 2ml/well were plated in 6-well plates. Plates were incubated for 24 h at 37°C in 5% CO_2. Subsequently, cells were transfected with the pΔElspIB Hex TK plasmid as described above. 6 h after the transfection, cells were treated with the drug gancyclovir at concentrations of 10 or 25 ug/ml. Each condition was done in triplicate. Cells survival was calculated in the treated population as a percentage of controls. Controls are cells transfected with the plasmid alone or treated with the drug alone. MTT assays was performed two days following treatment. The formazan crystals were dissolved in dimethyl sulfide (Fisher) and glycine buffer (0.1 M glycine- 0.1 M NaCl, pH 10.5). The formazan product formed by viable cells was quantitated by measuring the absorbance at a wavelength of 570 nm.

Results

Cell survival in the transfectants exposed to gancyclovir (GCV) at doses of 10 or 25 ug/ml was 50%

less than control cells treated with GCV alone or transfected with the plasmid only. We are presently examining the potential use of Hex II-VTK in recombinant Ad5 in the treatment of tumor bearing animals.

The regulation of this promoter in human tumor cell lines was studied using glucose, insulin, and glucagon. Lack of metabolic repression was confirmed as described by Mathupala, S.P. et al. ((1995) *J. Biol. Chem.* **270**, 16918-16925). In addition, several samples of human tissues were screened with the HK I, HK II, and HK IV cDNAs to evaluate the level of these enzymes in tissues and asses the safety of using this promoter in gene therapy.

We hypothesize that the Hex II promoter, with or without the metabolic manipulation of the normally express enzyme in muscle using glucagon will provide and important degree of selectivity to the anti-tumor effect. This represents a novel use of selective promoter, taking advantage of its abnormal regulation in tumor cells.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

In vivo localization of gene distribution and expression

The pΔE1spIBHex-LacZ may be used in tumor bearing rats for the *in vivo* localization of the suicide gene in pre-clinical testing of this novel targeting strategy. The gene construct is going to be administered in adenovirus type 5 recombinant vector or in lipid-based delivery system.

Materials and methods

Construction of recombinant viruses

Recombinant, replication deficient adenoviral vectors derived from type 5 adenovirus are constructed by the homologous recombination method in the human embryonic kidney cell line 293. The recombinant shuttle plasmids and pBHG11, containing the adenoviral genome, are co-transfected by calcium phosphate precipitation in 293 cells. The viral DNA is isolated from a single plaque and analyzed by restriction enzyme digestion. Recombinant adenovirus is expanded from a single plaque in 293 cells. Large scale production of the recombinant adenovirus is accomplished by growth in 293 spinner cells and purification by double cesium chloride gradient.

Results

These experiments are crucial to determine the best method of administration of the gene construct. It can either be done regionally to target specific organs such as the liver through portal vein injection or it can be administered intravenously. This method of looking at the distribution of the gene will allow us to determine the efficacy of uptake in the various organs and therefore establish a standard for use in humans.

EXAMPLE II

Targeted gene therapy for suicide destruction of tumors

The essential point is that the above-described HexII/VTK construct will be used in a vector/delivery system in clinical trials eventually.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any varia-

tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II promoter.

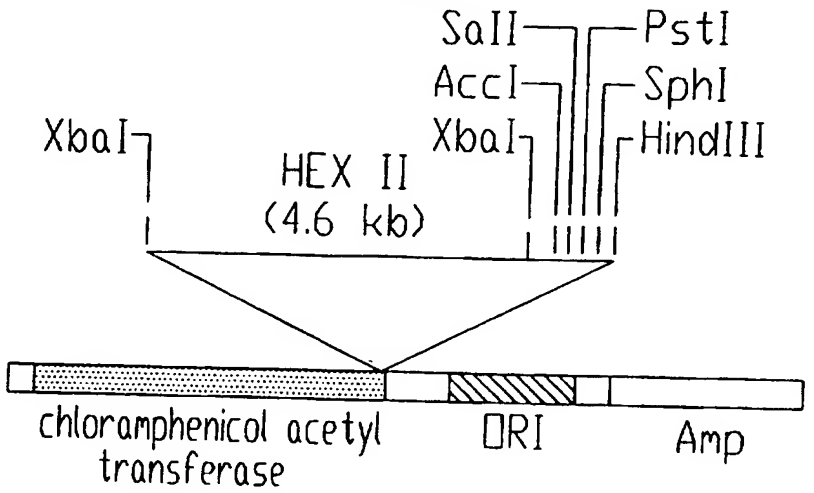
2. A Hex II gene construct, which comprises Hex II promoter in a vector selected from pCAT basic expression vector pΔElsplB and a shuttle plasmid.

3. The gene construct of claim 2, which further comprises β-gal or HSV Tk.

4. The gene construct of claim 2, wherein said vector is pCAT and said construct is pHexII4557-CAT.

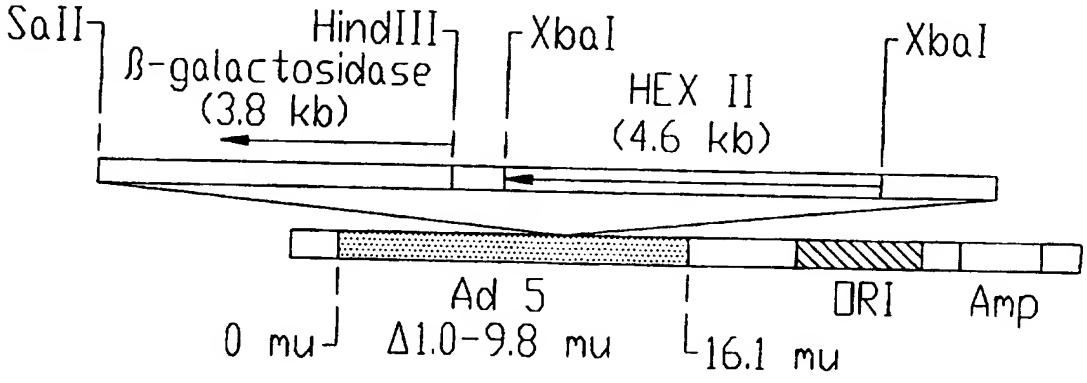
5. The gene construct of claim 3, wherein said vector is pΔElsplB and said construct is pΔElsplBHex-LacZ.

6. The gene construct of claim 3, wherein said vector is pΔElsplB and said construct is pΔElsplBHex-TK.



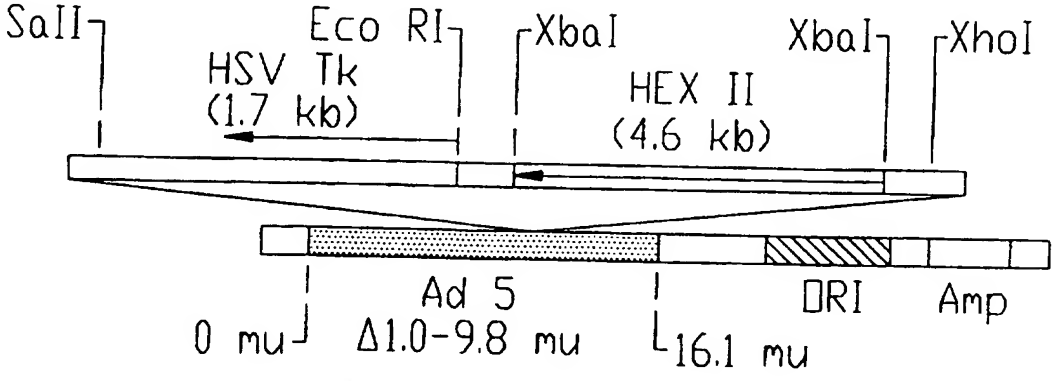
pHexII 4557/CAT

Fig - 1



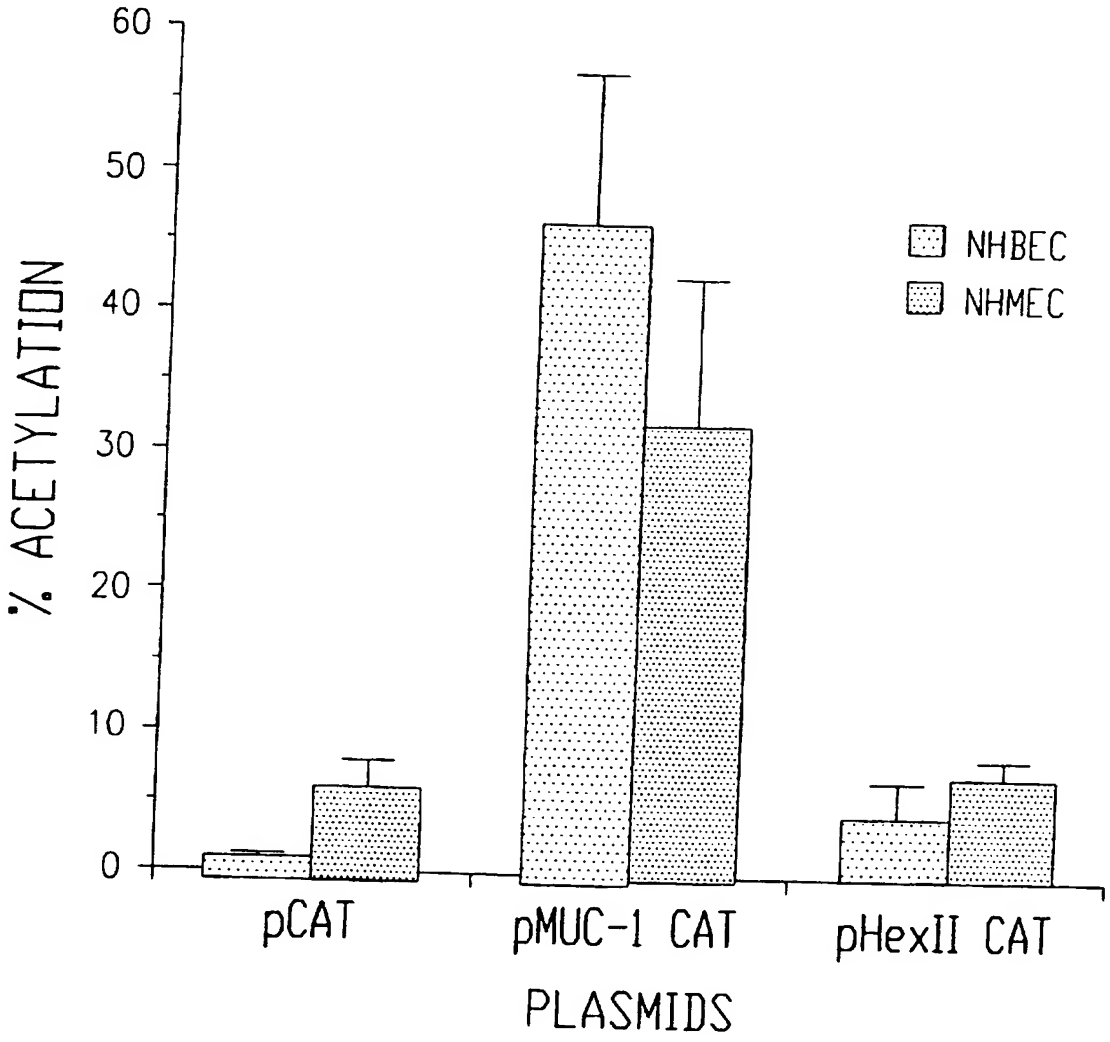
pΔE1sp1B HexII ß-galactosidase

Fig - 2



pΔE1sp1B HexII Tk

Fig - 3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50
40
30
20
10
0
% ACETYLATION
NHBEC
NHMEC
pCAT
pMUC-1 CAT
pHexII CAT
PLASMIDS

Fig. 4

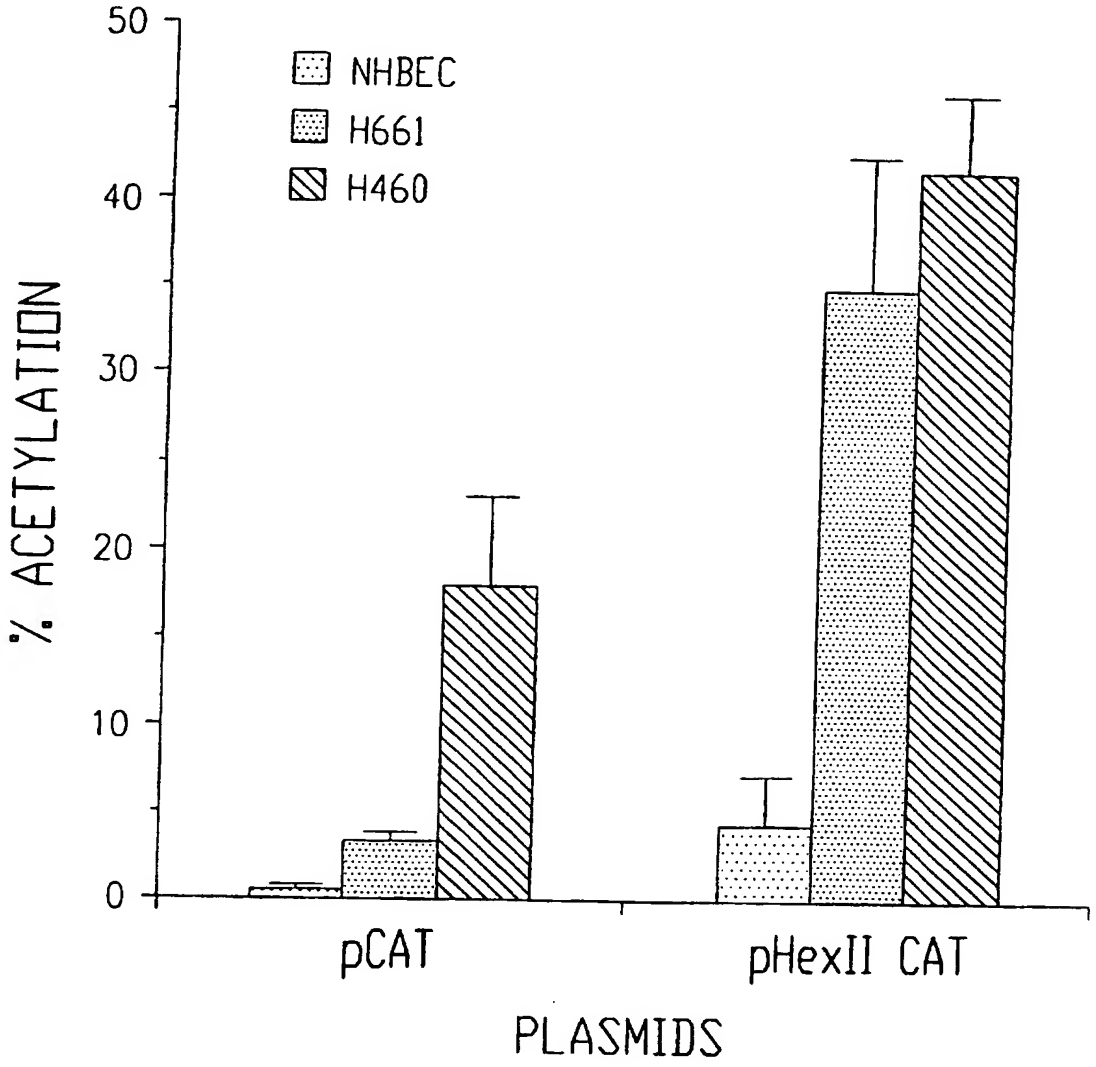

Fig 5

INTERNATIONAL SEARCH REPO

Ir tional Application No
PCT/CA 97/00691

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/54 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATHUPALA S. ET AL.: "Glucose catabolism in cancer cells" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 28, 14 July 1995, pages 16918-16925, XP002017888 cited in the application see the whole document ---	1
X	OSAWA H. ET AL.: "Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat hexokinase II gene" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 29, 19 July 1996, pages 17296-17303, XP002050907 see the whole document ---	1-5
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 December 1997	15/01/1998

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
A	REMPEL A. ET AL.: "Glucose metabolism in cancer cells: regulation of the Type II hexokinase promoter by glucose and cyclic AMP" FEBS LETTERS, vol. 385, no. 3, 6 May 1996, pages 233-237, XP002017889 see the whole document ---	1-6
A	HUBER B E ET AL: "VIRUS-DIRECTED ENZYME/PRODRUG THERAPY (VDEPT) SELECTIVELY ENGINEERING DRUG SENSITIVITY INTO TUMORS" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 716, 31 May 1994, pages 104-114, XP000654773 see the whole document ---	1-6
A	HARRIS J D ET AL: "GENE THERAPY FOR CANCER USING TUMOUR-SPECIFIC PRODRUG ACTIVATION" GENE THERAPY, vol. 1, no. 3, May 1994, pages 170-175, XP000654731 see the whole document ---	1-6
P,X	WO 97 04104 A (UNIV JOHNS HOPKINS ;PEDERSEN PETER LYNN (US); MATHUPALA SAROJ P (U) 6 February 1997 * see the whole document, esp. pp. 18-20, ex. 9 * -----	1-6

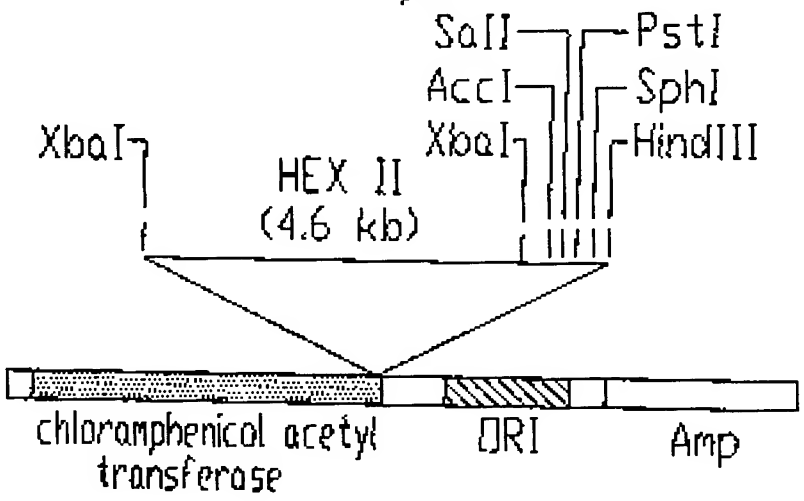
INTERNATIONAL SEARCH REPORT

nformation on patent family members

Int ional Application No
PCT/CA 97/00691

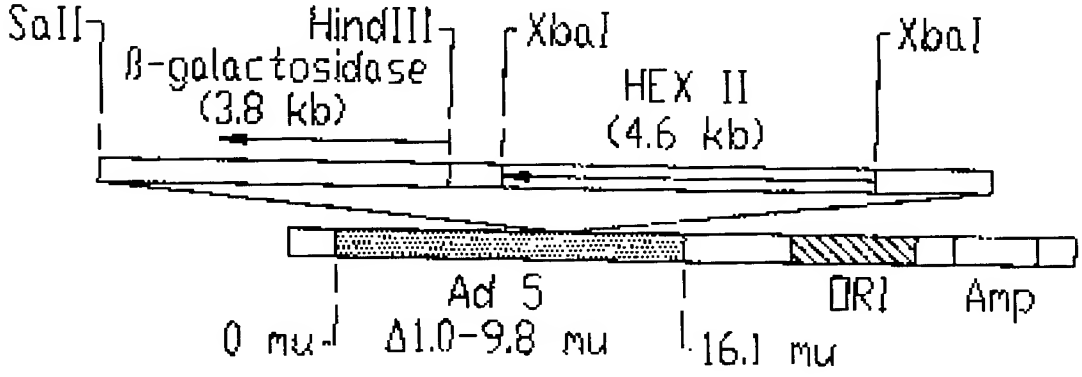
Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9704104 A	06-02-97	AU 6676396 A	18-02-97

Form PCT/ISA/210 (patent family annex) (July 1992)



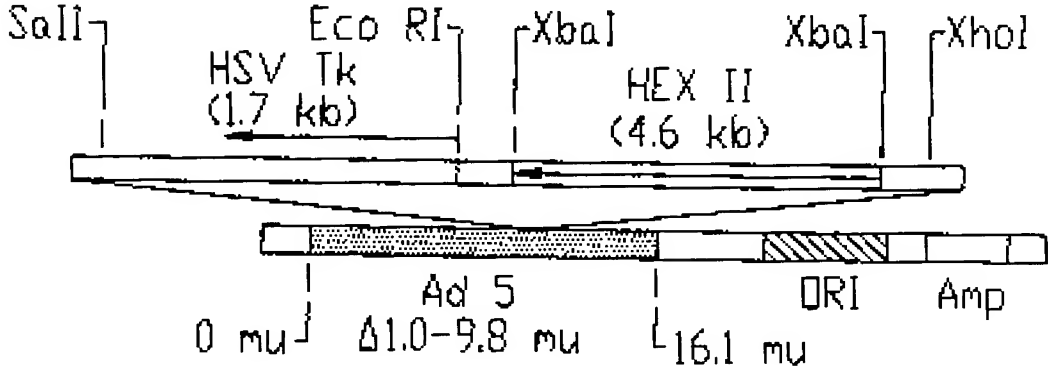
pHexII 4557/CAT

Fig - 1



pΔE1spIB HexII ß-galactosidase

Fig - 2



pΔE1spIB HexII Tk

Fig - 3

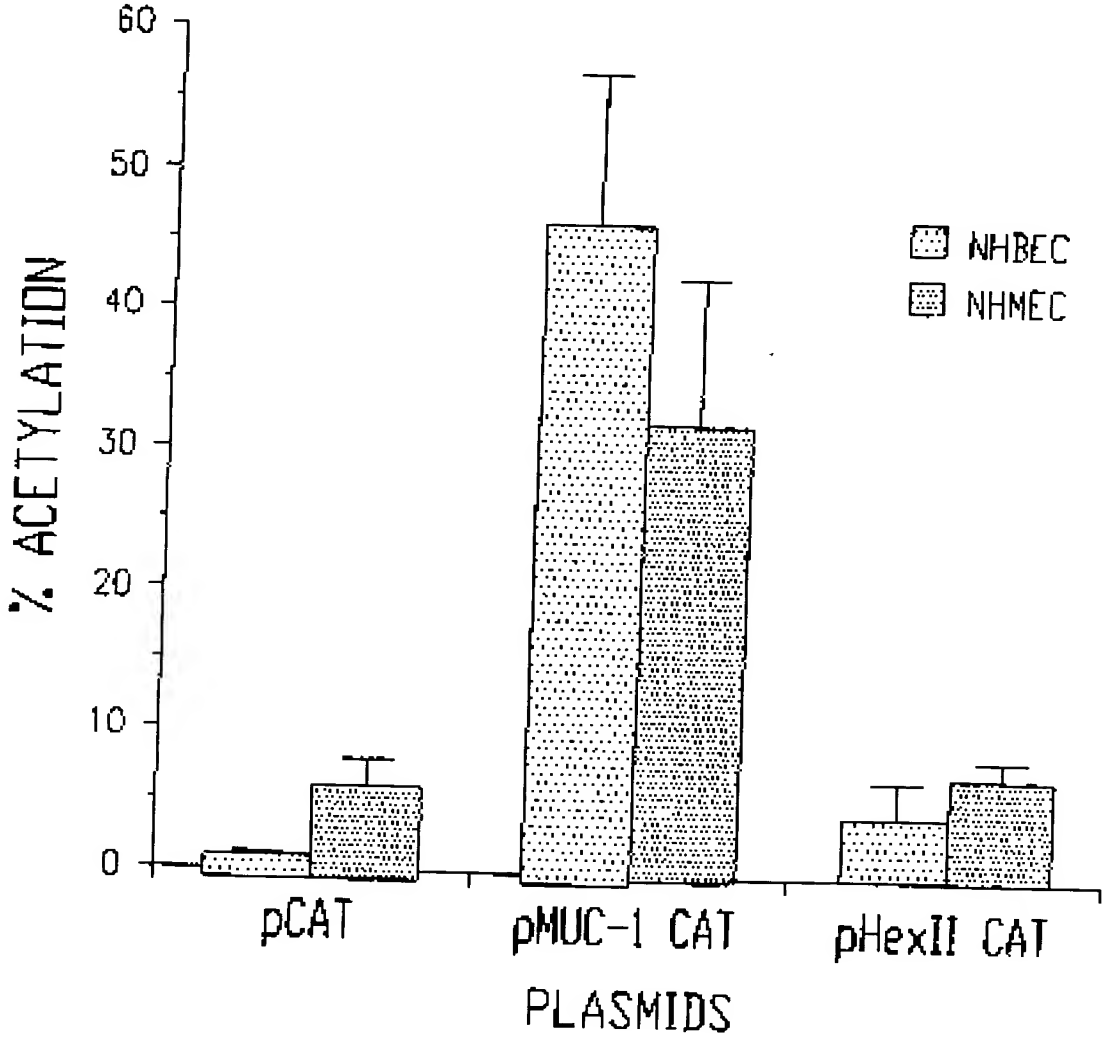

60
50
40
30
20
10
0
% ACETYLATION
NHBEC
NHMEC
pCAT
pMUC-1 CAT
pHexII CAT
PLASMIDS

Fig. 4

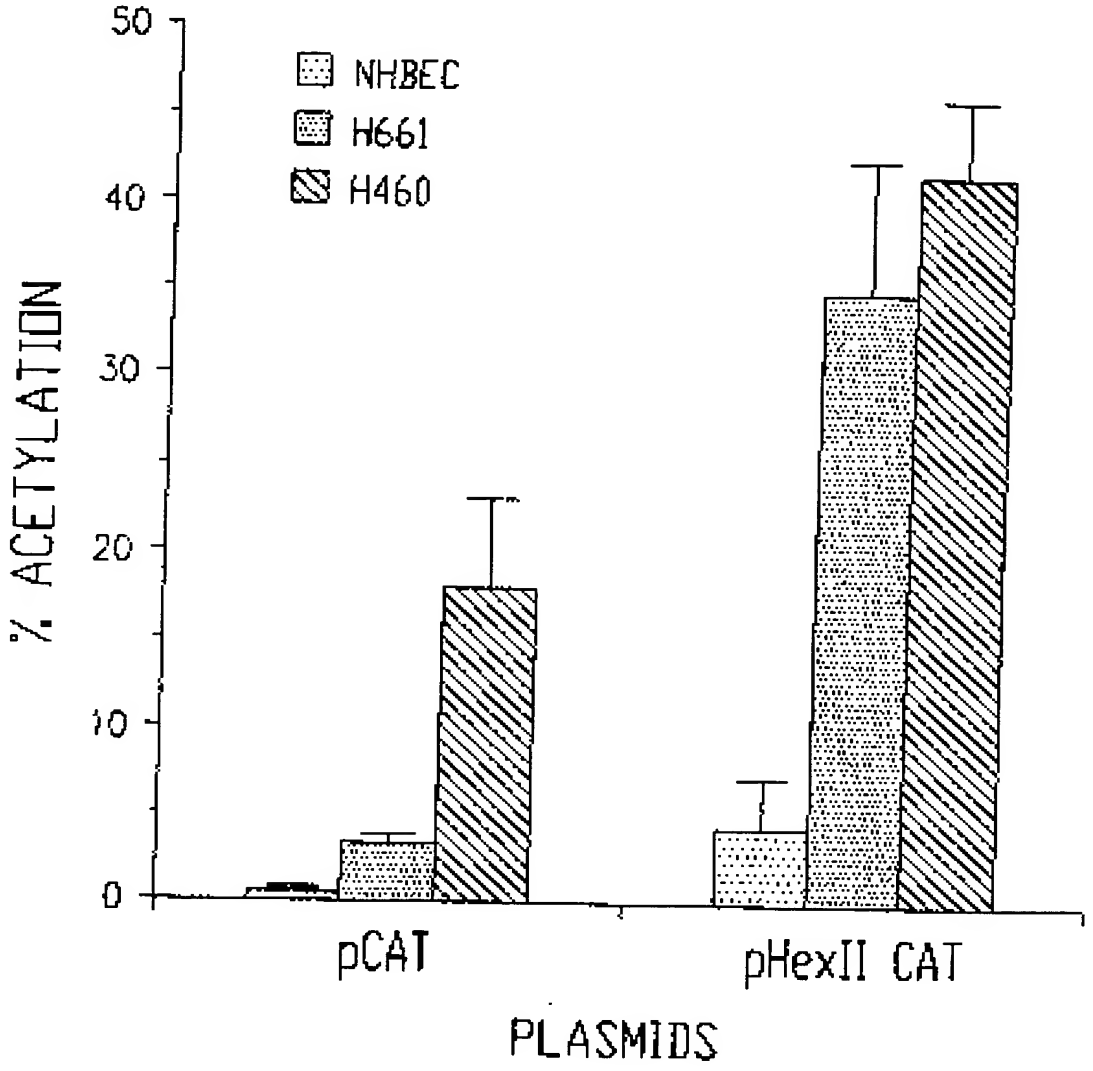


Fig. 5

RA P140 127

PATENT COOPERATION TREATY

ONTVANGEN

28 AUG 2001

AMERSFOORT

We

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

Prelexgum 1-10-2001 (PCT Rule 44.1)

NRF 1-11-2001

To:
VEREENIGDE
Attn. Prins, A.W., c.s.
Nieuwe Parklaan 97
2587 BN Den Haag
NETHERLANDS

Kopie in/naar

TERMIJN
23 AUG 2001

Beantwoord Voort. def.

Bericht gezonden aan dd.

MAP

Applicant's or agent's file reference P52075PC00	Date of mailing (day/month/year) 20/08/2001
	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/NL 01/00166	International filing date (day/month/year) 28/02/2001
Applicant RIJKSUNIVERSITEIT GRONINGEN	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41–22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL–2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Renate Jordan

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PCT/NL01/0016

WE

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
PRINS, A., W.
c/o Vereenigde
Nieuwe Parklaan 97
NL-2587 BN The Hague
PAYS-BAS

NRF2 1-11-2001

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Kopie in/naar | TERMIJN
0 9 OKT 2001

Beantwoord Voort. def. MAP | Behandeld gezonden dd.

Date of mailing (day/month/year)
27 September 2001 (27.09.01)

Applicant's or agent's file reference
P52075PC00

IMPORTANT NOTICE

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NL01/00166	28 February 2001 (28.02.01)	01 March 2000 (01.03.00)

Applicant
RIJKSUNIVERSITEIT GRONINGEN et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AG,AL,AM,AP,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EA,EE, EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD, MG,MK,MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   27 September 2001 (27.09.01) under No. WO 01/71015

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
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(25) Filing Language: English

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(30) Priority Data:
00200728.4 1 March 2000 (01.03.2000) EP

(71) Applicant *(for all designated States except US)*: **RIJKSUNIVERSITEIT GRONINGEN** [NL/NL]; Broerstraat 5, NL-9712 CP Groningen (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE LEIJ, Lou, Franciscus, Maria, Hubertus** [NL/NL]; Mooiland 77, NL-9734 BW Groningen (NL). **RUITERS, Marcel, Herman, Josef** [NL/NL]; M.L. Kingstraat 119, NL-9728 WN Groningen (NL). **MCLAUGHLIN, Pamela, Marijke, Jane** [NL/NL]; Westerbinnensingel 14, NL-9718 BT Groningen (NL). **HARMSEN, Martin, Conrad** [NL/NL]; Zevenhuizerweg 17, NL-9761 AD Eelde (NL). **VAN DER MOLEN, Henk** [NL/NL]; Ruischerwaard 29, NL-9734 CE Groningen (NL). **TERPSTRA, Peter** [NL/NL]; Marsstraat 73, NL-9743 EL Groningen (NL). **DOKTER, Willem, Hendrik, Abraham** [NL/NL]; Van Beethovenstraat 65, NL-6521 EL Nijmegen (NL).

(74) Agent: **PRINS, A., W.**; c/o Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/71015 A2

(54) Title: NON-SQUAMOUS EPITHELIUM-SPECIFIC TRANSCRIPTION

(57) Abstract: The invention relates to the field of cancer therapy and diagnosis, in particular of carcinomas. The invention provides an isolated and/or recombinant nucleic acid comprising a tissue specific promoter or functional fragment thereof allowing for expression of a nucleic acid of interest operably linked to said promoter or functional fragment thereof in a cancer cell wherein said expression in said cancer cell is essentially carcinoma selective.

Title: Non-squamous epithelium-specific transcription.

The invention relates to the field of cancer therapy and diagnosis, in particular of carcinomas.

Carcinomas, as for example distinguishable from sarcomas, lymphomas, or melanomas, in general are the malignant counterparts or neoplasia derived from epithelia. Distinct carcinoma types are basal cell or squamous cell carcinoma of the oral, laryngeal or oesophageal mucosae; carcinomas of the intestines, such as gastric adenocarcinoma, small intestinal or colorectal carcinoma; cholangiocarcinoma; pancreatic carcinoma, lung carcinoma; prostate, testicular, mammary, cervical, ovarian and endometrial carcinoma, and so on.

Carcinomas are in general a difficult tumour-type to treat. Despite numerous improvements in radiological, chemo-therapeutical and surgical techniques current treatments for metastatic malignant disease such as carcinomas are often ineffective. Therefore, new immuno-therapeutical and genetic strategies, which can enhance the selectivity of systemic therapy so that tumour response is increased without toxicity to normal tissue, have gained interest. For example, various tumour antigens, such as the human pancarcinoma associated epithelial glycoprotein-2 (EGP-2), also referred to as 17-1A or Ep-CAM, have been a target for immunomodulation. Antibodies to EGP-2, a transmembrane antigen, have been successfully used in patients for imaging of small cell lung cancer and for adjuvant treatment of minimal residual disease of colon carcinoma leading to an increased survival of this otherwise poorly prognosed disease. By reducing the size of antibodies to create so-called ScFVs, by humanising constant regions to lower the immunogenicity, by designing bispecific constructs to bring immune effector cells into contact with tumour cells, by fusing antibodies to cytokines, drugs, or gene delivery vehicles, or by developing vaccines to tumour antigens, a number of groups have enhanced the potential of anti-tumour antigen mediated immunotherapy. However, still efficacy of immuno-therapy has to be evaluated, and effective animal models for such evaluation are often lacking.

Another promising strategy against cancer is genetic prodrug activation therapy which aims to use differences between normal and neoplastic cells to drive the selective expression of a metabolic suicide gene that is able to convert a nontoxic prodrug into its toxic metabolite. A well known suicide gene strategy comprises the

use of thymidine kinase gene and for example gancyclovir. However, despite the availability of promising gene/prodrug systems a major impediment to the development of gene therapy treatments is the lack of suitable expression cassettes for directing selective transgene expression. In particular there is little or no information on how to achieve carcinoma specific or selective transgene expression.

The invention provides an isolated and/or recombinant nucleic acid comprising a tissue specific promoter, promoter/enhancer, or functional fragment thereof allowing for expression of a nucleic acid of interest operably linked to said promoter or functional fragment thereof in a cancer cell wherein said expression in said cancer cell is essentially epithelia or carcinoma selective in that expression in carcinoma related cancer or epithelial cells is clearly more prominent than expression in non-carcinoma related cancer or epithelial cells. Of course, its expression being essentially carcinoma-selective also has impact on its expression pattern in non-cancer cells. In one embodiment, the invention provides a nucleic acid comprising said promoter or functional fragment thereof allowing for mainly simple or non-squamous epithelium-specific expression in essentially adult or well-differentiated tissue, at least in tissue developed beyond the stage of embryonic development, of said nucleic acid of interest operably linked to said promoter or functional fragment thereof, thereby differentiating a promoter or functional fragment thereof from for example a tissue specific human keratin 18 promoter (Abe and Oshima, JCB 111:1197-1206) which includes hepatocytes, and thus avoiding the possibility of liver-failure due to tissue-specific transgene expression of for example a suicide gene regulated by such a K18 promoter.

Also, the promoter provided herein clearly differs from for example the hexokinase II gene promoter to drive the of a expression suicide gene in tumors (WO 98/13507) which is not is not restricted to or selective for carcinomas, but extends to be expressed in all tumor cells and beyond. In vitro, the expression of hexokinase II in tumor cells, such as AH310 hepatoma and HepG2, was, albeit higher than in normal cells, i.e. hepatocytes, not-selective for epithelial cancer cells. Consequently, suicide gene therapy, utilizing said promoter against a plethora of tumor types carries the intrinsic potential severe side effect of killing the normal counterpart cell type of which the tumor was derived, because many cell types express hexokinase II.

Furthermore, several of the normal cell types will often be readily accessible for the gene therapeutic device, given that a "foolproof" gene-targeting vectorsystem has to be developed yet, whereas a promoter as provided herein requires little or no targeting.

Similarly, the use of TNF-alpha to manipulate the expression of PKR (double-stranded RNA dependent protein kinase) with the aim to induce in apoptosis in tumor cells (WO 98/00013) is not restricted to carcinomas, but for example also shown to be the case for CEA expressing tumors, hepatoma, and Kaposi's sarcoma, which are clearly not of epithelial descent. Several of the TNF-induced promoters are known to show at least low levels of transcription in normal tissue cells, again presenting the risk of increased side effects (increased apoptosis in normal tissues). In addition, several tumor cell lines show decreased responsiveness to TNF-alpha in vitro and are thus less prone to apoptosis. In addition, vascular endothelial cells are sensitive to TNF-alpha and are in constant contact with the blood stream. Therefore, the risk exists that the PKR constructs could be taken up by these endothial cells and subsequently express low levels of the enzyme. This causes local apoptotic lesions, which may present as vascular leakage (oedema) or other endothelial disturbances such as coagulation alterations.

An advantage of treating carcinomas as provided herein, especially using suicide gene-therapy, not only resides in the enhanced specificity (selectivity) for corcinomas, but also resides for example in the functional barrier formed by a basal membrane behind which the normal epithelial cells are located. In contrast, the carcinoma cells comprise a much more loose tissue, which not only is strongly vascularized, but is much more accessible for large (targeted) molecules.

In a preferred embodiment, the invention provides a promoter or functional fragment thereof derived from a EGP-2 gene. Being a so-called pan-carcinoma associated antigen makes the human epithelial glycoprotein-2 (EGP-2) suitable for use as target for immuno therapy and gene therapy strategies. Defined by for example the Mabs CO17-1, GA733-2, MOC-31 and 323/A3 this kb EGP-2 protein, also referred to as Ep-CAM or 17-1A, is encoded by the GA733-2 gene. EGP-2 is expressed on all epithelial tissue derived cancers like that of the breast, pancreas, gonads, gastrointestinal, respiratory, and urinary tract, more benign epithelial neoplasias such as polyps often have high EGP-2 expression as well, whereas expression in

normal tissue is limited to the baso-lateral cell surface of simple epithelia. EGP-2 has been defined as a homophilic adhesion molecule, which expression is associated with active proliferation and a morphoregulatory role in organogenesis. Since the isolation of antibodies of EGP-2 in 1979, immunotherapeutical strategies using EGP-2 as a target have been developed and are at present, with limited success, used in clinical settings. Use of the EGP-2 protein's carcinoma specificity for the development of gene therapy strategies, however, has, until now, never been possible considering the fact that the regulatory sequences directing this specificity could not be isolated and characterised. Here we provide the isolation and characterisation of the 5' sequences from the GA733-2 gene and the identification of (preferably cis-acting) sequences needed for selective expression of heterologous genes in EGP-2 positive cells or cells functionally equivalent thereto, such as (non-human) animal cells expressing EPG-2 functional homologues. Expression of a nucleic acid of interest operably linked to a promoter or functional fragment thereof as provided by the invention is thus mainly restricted to normal adult non-squamous epithelium or neoplasias derived from epithelia, such as wherein said epithelium comprises lung, kidney, pancreas, testis, bile duct or intestinal epithelium and other not yet defined neoplasias derived thereof comprising carcinoma cells.

In a preferred embodiment, the invention provides an isolated and/or recombinant nucleic acid comprising a tissue specific promoter, promoter/enhancer, or functional fragment thereof allowing for expression of a nucleic acid of interest operably linked to said promoter or functional fragment thereof wherein said carcinoma cells comprise lung carcinoma cells.

In a much preferred embodiment, the invention provides a nucleic acid according to the invention comprising a nucleic acid or functional fragment thereof as shown in figure 1, in particular the invention provides a nucleic acid fragment as shown from about position -778 to about position -422, or as shown from about position -1113 to about position -422, or as from about position -2190 to about position -422, or from about position -778 to about position 0, or further extensions thereof, as for example shown in figure 1, where a tissue-specific functional fragment is given or a nucleic acid functionally equivalent thereto, said functional equivalent preferably comprising the necessary epithelial transcription sites to render the fragment tissue-specific.

In a preferred embodiment, said promoter or functional fragment thereof does not comprise a canonical TATA box or an atypical CAAT box, as for example is the case with most promoter sequences such as for example with GA733-1 promoter sequences (see Linnenbach et al., PNAS 86:27-31, 1989; also as discussed in Siemieniako and Wiland, Biochem. Biophys, Res. Comm. 186:1353-1361, however, providing a promoter or functional fragment or equivalent thereof according to the invention with such a box or boxes does not necessarily deprive it of its tissue specific nature.

In a preferred embodiment, the invention provides a nucleic acid derived from a mammal, such as an experimental animal as a mouse or rat, or, preferably derived from a human.

The invention further provides a nucleic acid according to the invention further comprising a nucleic acid of interest. For example, fusions of the EGP-2 promoter (if desired the 55 kb XhoI-XhoI fragment comprising an EGP-2 genomic region or functional fragments thereof such as a smaller 10kb 5'-end fragment or approx. 3.5kb *Xma*III 5'-end fragment, see sites therein in fig.1) with nucleic acid encoding heterologous protein(s) are provided to drive its expression in a epithelium specific fashion. Such a nucleic acid of interest can for example be a reporter gene, or functional fragment thereof, such as a GFP or luciferase gene, for example providing candidate drug tests wherein compounds are screened for their activity to regulate or modulate promoter/enhancer sequences according to the invention, preferably in a tissue specific way.

Another example comprises a nucleic acid according to the invention further comprising an inducable or suppressible promoter or functional fragment thereof.

The invention also provides a nucleic acid according to the invention further comprising a suicide gene or functional fragment thereof. Several suicides genes are known and can be applied, in one embodiment of the invention, said suicide gene comprises a non-mammalian cytosine deaminase (CD) gene. Genetically modified cells that express the nonmammalian enzyme cytosine deaminase (CD) gene are able to convert the nontoxic prodrug fluorocytosine (5-FC) to the toxic metabolite fluorouracil (5-FU). 5-FU inhibits DNA synthesis during the S phase of the cell cycle. In addition to direct cytotoxicity to the transfected cells significant toxicity from the converted prodrug can be transmitted to adjacent cells. This gene/prodrug system can

even compensate for the inability of vector systems that cannot transfect all cells of a tumor. In a preferred embodiment, the invention provides use of EGP-2 transcriptional regulatory sequences to regulate transient expression of the cytosine deaminase (CD) gene in EGP-2 expressing carcinoma cells. CD expression using these constructs correlated well with the expression of endogenous EGP-2 and demonstrated effective killing of the EGP-2 positive cells.

The invention furthermore provides a vector, such as a plasmid, clone, or gene delivery vehicle, such as a non-viral or viral vector, such as an adenovirus vector, comprising a nucleic acid according to the invention. Such gene delivery vehicles as provided by the invention are very useful in carcinoma therapy, or in therapy directed at non-squamous normal epithelial disease.

The invention for example provides a gene delivery vehicle according to the invention targeted to carcinoma cells which is useful for tumour-selective suicide gene therapy, such as a vector provided with an EPG-2/CD chimeric gene construct as provided herein, optionally, when so desired, to be used in combination with a pan-carcinoma specific (for example an EGP-2-specific) gene delivery system enhancing the safety and efficacy of vector based anti-cancer gene therapy approaches according to the invention even further.

In addition, the invention provides a host cell comprising a nucleic acid, a vector or a gene delivery vehicle according to the invention, such host cells for example being oocytes wherein a nucleic acid according to the invention has been introduced to generate an transgenic cell, cell-line or animal, but such host cells also providing target cells for candidate drug tests wherein compounds are screened for their activity to regulate or modulate promoter/enhancer sequences according to the invention, preferably in a tissue specific way, thereby allowing detection of carcinoma specific drugs from amongst a great variety of compounds, preferably having been derived at by combinatorial chemistry.

Furthermore, the invention provides an experimental animal comprising a cell according to the invention, such as for example derived from an oocyte as provided by the invention. The invention provides for example the isolation of the EGP-2 regulatory sequences and usage of these sequences to direct epithelial specific EGP-2 expression in mice in fashion with the situation in humans. In this EGP-2 expressing mouse model as provided by the invention EGP-2-specific tolerance was observed.

The invention furthermore provides a method for evaluating a possible treatment of disease comprising testing such treatment on a host cell or an animal according to the invention, in particular wherein said treatment comprises treatment of disease comprising non-squamous epithelium or wherein said disease comprises carcinogenesis. For example, the EGP-2 transgenic host cell and mouse provided here may serve as a model to study the biology of the EGP-2 molecule and to evaluate efficacy and safety of the variety of generated anti-EGP-2 based immunotherapeutical modalities influencing diseases. Not only evaluation of immune therapy is provided, said mice can also serve to evaluate gene therapy, especially gene therapy aimed at treatment of carcinomas, or serve to further evaluate drug treatment. Cells, cell-lines and animals, such as rats or mice as provided by the invention can also be used for carcinogenicity testing, for example by providing a nucleic acid comprising promoter or functional fragment thereof according to the invention with an additional nucleic acid of interest such as a nucleic acid encoding the large T antigen of the SV40 virus, or any other protein or fragment thereof involved in (the onset of) tumourgenesis, whereby a cancer prone host cell or experimental animal is provided, specifically suited for detecting carcinogenicity by detecting carcinoma development. Transgenic animals generated with such a construct develop spontaneous tumors derived from epithelial tissues as only in these tissues the large T antigen is expressed.

In another example, the EGP-2 promoter sequences are fused to generate epithelium tissue specific deleter mice which can be used to create epithelium specific gene knock-out mice. For instance, fusion of the EGP-2 promoter with coding sequences of the *cre* recombinase is provided for the generation of transgenic mice. This yields a *deleter* line with which it is possible to generate knockout mice that specifically lack expression of the "knocked out" gene in epithelial tissues, provided this gene had *lox*P sites to enable the *cre* recombinase to excise the gene. Similarly, the combination of the EGP-2 promoter with the *Cre*-ER^T fusion protein generates *deleter* mice in which the epithelium specific deletion of *lox*P insertions can be induced with 4-hydroxy-tamoxifen. The *Cre*-ER^T protein is a tamoxifen-dependent derivative of the "normal" *cre* recombinase (R.Feil, J.Brocard, B.Mascrez, M.LeMeur, D.Metzger and P.Chambon (1996) PNAS 93:10887-90).

Also, a fusion of the EGP-2 promoter with the GFP (green fluorescent protein, or any other reporter protein) is provided to generate transgenic animals. Preferably

this is the $p39^E$ construct that was used to determine to minimal epithelium specific promoter. These transgenic animals express a reporter such as GFP in an epithelium specific manner. The use in organ transplant models enable to study the fate of donor derived epithelial cells. This is an important topic in transplantation science, as it will shed new light on issues that concern transplant rejection.

The invention furthermore provides use of a nucleic acid, a vector or a gene delivery vehicle according to invention for the preparation of a medicament, in particular wherein said medicament is for the treatment of cancer, preferably a carcinoma. Such a medicament as provided by the invention may comprise a nucleic acid, a vector or a gene delivery vehicle according to the invention, but may also be derived from a drug found in a candidate drug test as identified above. Said medicaments find their use in a method for the treatment of cancer as provided by the invention, for example comprising administering to a patient a nucleic acid, a vector, a gene delivery vehicle or a drug according the invention.

The invention is further described in the detailed description without limiting the invention thereto.

Detailed description

Based on immunohistochemical data, EGP-2 mainly shows expression in normal adult and fetal epithelial tissues, by most it is even seen as a strictly non-squamous epithelial molecule in adult humans (Balzar et al., J. Mol. Med. 77:699-712, 1999). EGP-2 is detected at the basolateral cell membrane of all simple (especially glandular), pseudo-stratified, and transitional epithelia. In contrast, normal squamous stratified epithelia are negative for EGP-2. In adult human tissues no expression was found in mesenchymal, muscular, brain and neural tissues. Furthermore, no EGP-2 expression was detected in cells of lymphoid origin. The level of expression may differ significantly between the individual tissue types. In the gastro-intestinal tract, the gastric epithelium expresses very low levels of EGP-2. Expression levels are substantially higher in small intestine, and in colon EGP-2 is probably expressed at the highest levels among all epithelial cell types. Glandular epithelium of the gall-bladder express EGP-2 but the transitional epithelium (urothelium) of the bladder is only slightly positive. In the lower respiratory tract,

bronchi, bronchioles, and alveoli are EGP-2 positive. In adult liver the bile ducts are EGP-2 positive, whereas hepatocytes are negative. Most epithelial cells of the kidney, such as cells of the proximal tubules, distal tubules, and ducts, express EGP-2. In pancreas EGP-2 expression has been detected in the ductal epithelium and acini. In skin, the sweat ducts and the proliferative zone of the hair follicle reveal EGP-2 staining, whereas keratinocytes and melanocytes are essentially negative. Within the basal layers of the epidermis some EGP-2 reactivity can be observed in the reserve cells, since mAb MH99 was reported to be reactive with some cells within the basal layer of skin keratinocytes. The glands of the endocrine system (thyroid, parathyroid, pituitary and adrenal glands) contain EGP-2-positive epithelium. In mammary glands, the ductal epithelium reveals relatively high levels of EGP-2 expression. EGP-2 expression is detected in most epithelial tissues of the female genital tract (ovaries, oviducts, cervix, and uterus). Normal endocervical glandular epithelium (both columnar and reserve cells) reveals high expression levels of EGP-2, whereas ectocervical squamous epithelial cells do not express the molecule. Some EGP-2 expression may be detected in the basal cells of morphologically normal ectocervical tissue, but only in areas bordering lesions of cervical intra- epithelial neoplasia. In tissues of the male genital tract, some of the epithelial cells in testis, epididymis, seminal vesicle, and prostate reveal EGP-2 expression.

EGP-2 is a marker for differential diagnosis and prognosis of several types of carcinomas. Active proliferation in a number of epithelial tissues is associated with increased or de novo EGP-2 expression. This is especially evident in tissues that normally reveal no or low levels of EGP-2 expression, such as squamous epithelium. At the early stages of neoplasias of the uterine cervix, de novo expression of EGP-2 is often observed in areas with atypical, undifferentiated cells of the squamous epithelium. Thus, in cervical intraepithelial neoplasia (CIN) grades I and II, the basal and suprabasal cells are EGP-2 positive, while grade III lesions reveal up to 100% positive cells in all layers of the squamous epithelium. Moreover, a clear increase in both the number of positive cells and the level of EGP-2 expression is observed during the progression from CIN I to CIN III. Expression of EGP-2 in atypical cells of CIN lesions correlated with the disappearance of markers for squamous differentiation and enhanced proliferation. In weak, mild and severe oral mucosal dysplasias high levels of EGP-2 expression were detected in basal and suprabasal cells with a clear

border between EGP-2-positive dysplastic cells and EGP-2-negative normal epithelial cells.

In glandular epithelium of the gastrointestinal tract, one can observe a gradient of decreasing expression of EGP-2 from crypts to villae. The level of EGP-2 expression correlates with the proliferative activity of intestinal cells, and inversely correlates with their differentiation. Dysplastic or metaplastic proliferation corresponds to an increase (sometimes to very high levels) in EGP-2 expression. In gastric epithelium that normally expresses low levels of EGP-2, a strong expression of EGP-2 is observed in proliferative metaplastic lesions, such as intestinal metaplasia. Even in colon, where the epithelium expresses very high levels of EGP-2, the development of polyps is reported to be associated with an increased expression of the molecule. Hepatocytes are EGP-2-positive during embryonic development (week 8 embryos), but negative in adult liver. However, during liver regeneration processes cells that morphologically resemble precursor stem cells are EGP-2-positive, but, as they mature into hepatocytes, they again become EGP-2-negative. Dysplastic lesions of the bladder epithelium (urothelium) reveal increased EGP-2 expression as compared to normal urothelium.

In human tissue EGP-2 is expressed only in epithelium and neoplasias derived from epithelia. Therefore, the molecule may be used as a marker to distinguish epithelial neoplasias from neoplasias derived from non-epithelial tissues. EGP-2-positive tumors are derived from epithelial cells, whereas EGP-2-negative tumors may originate from non-epithelial as well as epithelial tissues. Furthermore, EGP-2 may be used as a marker to histologically differentiate between epithelial neoplasias. Occasionally, difficulties in the histological differential diagnosis between basal-cell carcinoma (BCC) and squamous-cell carcinoma (SCC) of the skin may arise. Basal squamous cell epithelioma, a tumor combining morphological properties of BCC and SCC, is one common example of these difficulties, but other histological types of BCC may also be erroneously interpreted as SCC. Staining for EGP-2 demonstrated that all BCCs are diffusely and intensely labelled, whereas none of the SCCs expressed EGP-2, irrespective of the histological type or grade of differentiation. In liver neoplasias, EGP-2 was found to be expressed in almost all cholangiocarcinomas, whereas the majority of hepatocellular carcinomas were EGP-2-negative, suggesting that the hepatocellular carcinoma originates from a highly differentiated precursor.

The results also indicate that EGP-2 can be used as an additional immunohistochemical marker to distinguish cholangiocarcinoma from hepatocellular carcinoma due to the differential expression in these epithelial tumors. Finally, it was demonstrated that EGP-2 can be used as a marker to discriminate carcinomas from EGP-2-negative mesotheliomas, except for the epithelioid types.

Malignant proliferation is nearly always associated with EGP-2 expression at some stage of tumour development. Most carcinomas, but no other tumour types, express high levels of EGP-2. However, EGP-2 expression in carcinomas can be heterogeneous, and is probably affected by a shift of tumour cell differentiation to either mesenchymal or squamous (in squamous carcinomas) cell phenotypes. It has been reported for dysplastic oral mucosa that well-differentiated squamous cell carcinomas are negative for EGP-2, whereas poorly differentiated squamous cell carcinomas are EGP-2-positive. Most squamous carcinomas are EGP-2-positive, except for (EGP-2-negative) squamous carcinoma of the skin. The expression of EGP-2 distinguishes squamous cell carcinoma of the skin from the EGP-2-positive basal cell carcinoma. Varying levels of EGP-2 expression were detected in the majority of squamous and adenocarcinomas of the uterine cervix.

The EGP-2 antigen has been suggested to be a homophilic adhesion protein, but the function of this protein is poorly understood (M. Trebuk et al., JBC (2001) 276 (3) 2299-2309). Transgenic mice have been used extensively to determine the function of proteins both in the development of diseases as for the evaluation of anti-disease therapies. However, although cloned in 1990, no suitable transgenic animal model expressing EGP-2 has been generated thus far, probably as a result of the fact that no appropriate regulatory sequences were available. Besides being expressed on most carcinomas, EGP-2 is also expressed on the baso-lateral cell surface of simple, transitional, and pseudostratified epithelia of the respiratory, gastrointestinal and urinary tract, the pancreas, gonads, and uterus/cervix, but not on hart, spleen, muscle, brain and connective tissue. When using EGP-2 as a target for immuno therapy there is a risk of side-effects induced by targeting to the antigen on this normal tissue. Indeed toxicity problems were observed after treatment with high-affinity anti-EGP-2 mAbs and a high affinity anti-EGP-2 mAb derived bispecific antibody. Thus, the relevance of an animal model to study immunotherapy targeting of the EGP-2 antigen for future use in patients is dependent on the expression of the

antigen on normal animal tissues. Endogenous EGP-2 expressed by the mouse itself has been used to study anti-EGP-2 immunotherapy strategies however the immunotherapeutic molecule evaluated in wild type mice can not be used as a therapeutic in patients. Although the overall distribution of mEGP-2 is similar to human EGP-2, mEGP-2 expression was additionally observed in lymphoid organs like spleen and thymus and T-, B-, and dendritic cells. So results obtained in wild-type mice using mEGP-2 as a target may not hold true for humans. Although a transgenic mouse model expressing EGP-2 under the control of the mouse mammary tumour virus promoter and a transgenic rat model expressing EGP-2 under the control of the human keratin 18 regulatory sequences have been generated previously, the EGP-2 expression pattern observed in these transgenic animals did not resemble the human expression pattern.

Materials and Methods

Cell Culture

Both the human SCLC derived cell lines GLC-1 (EGP-2 negative) and GLC-45 (EGP-2 positive) (De Leij,1985), as the human rectum adenocarcinoma cell line SW948 (EGP-2 positive) obtained from the ATCC (Rockville, MD) (CCL 237), were cultured according to routine procedures in complete medium, i.e. RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 50 µg/ml Gentamycine Sulfate; 2 mM L-Glutamin; 1mM Sodium Pyruvate (Gibco BRL); 0.05 mM β-mercaptoethanol (Biowhittaker) and 10% FCS (Bodinco) at 37°C in humidified 5% CO_2 atmosphere. The SV40 transformed simian kidney cell line COS-7 also obtained from the ATCC (CRL 1651) and the primary human fetal lung fibroblasts (FLF) were cultured in DMEM (Gibco BRL) supplemented with 50 µg/ml Gentamycine Sulfate (Biowhittaker); 2 mM L-Glutamin (Gibco BRL); 10% FCS at 37°C in humidified 5% CO_2 atmosphere. Endothelial cells were isolated from human umbilical veins (HUVEC) and cultured in RPMI 1640 supplemented with 20% heat-inactivated human serum, 2 mM L-Glutamin, 5 U/ml heparin , 50µg/ml EC growth factor , 100µg/ml streptomycin and 100 U/ml penicillin in 1% gelatin coated tissue culture flasks (Costar) at 37°C in humidified 5% CO_2.

Cell transfection:

The adherent cells were transfected by either the Saint (Saint Inc., Groningen, The Netherlands) or the FuGENE-6 (Boehringer-Mannheim, Dusseldorf, Germany) method. 1 day prior to transfection 6-wells plates (Costar) were seeded with 1-3 x 10^5 cells/well. The cells were transfected per well by 3 µl FuGENE-6 in 100 µl serum-free medium added to 0.5 µg DNA, which was subsequently dropwise added to the cells in standard culture medium. Or the cells were transfected, after washing twice with HBSS (Gibco BRL), with 33 µl 0.75 mM Saint in 100 µl HBSS added to 0.5 µg DNA in 100 µl HBSS completed to 1 ml with serum free medium of choice per well. After 3-4 h. incubation with this Saint-DNA serum free medium 2 ml standard culture medium was added. 24 h. irrespective of the transfection method used the cells were harvested by detaching them from the surface by trypsin/EDTA (0.5/0.2 mg/ml) in PBS and prepared for further analysis.

Isolation of the GA733-2 promoter region.

For the isolation of the GA733-2 5'sequences, a BAC genomic library was screened commercially by GenomeSystem, Inc. (St. Louis, Missouri, USA) with a 920 bps [^{32}P]-labeled genomic DNA fragment containing approximately 250 bp of the 5' region of the human EGP-2 gene GA733-2 in addition to the exons 1, 2, and 3. The probe was derived from the GA21726-22RS vector, kindly provided by Dr. Linnenbach (Wistar Institute, Philadelphia, USA), by digestion with *SalI*/*Sac*II. DNA from the one positive clone was purified according to standard methods for BAC DNA isolation and analyzed by restriction mapping and Southern blot analysis.

Southern blot analysis.

Since a *Sac*II restriction site was present 39 bp downstream of the ATG, digestions with either *Sac*II alone or in combination with; *Hind*III, *Eco*RI, *Bam*HI, *Pst*I, *Xba*I, *Bgl*II, *Eco*RV, *Sma*I, and *Xho*I (All obtained from Boehringer-Manheim) were carried out. After separation on a 0.8 % agarose gel the DNA was transferred to a hybond N+ nylon transfer membrane (Amersham, Bucks, UK) and subsequently hybridized with the above described [^{32}P]dCTP labeled *SalI*/*Sac*II EGP-2 promoter probe at 65°C for 18 h in 1 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2), and 7% SDS. Membranes were

washed once in 2× SSC, 0.1% SDS; once in 1× SSC, 0.1% SDS; 0.3× SSC, 0.1% for 1 h at 65°C and visualized and quantified by autoradiography. A 4.2 kb spanning *Sac*II/*Bgl*II genomic subfragment containing at least exon 1 and approximately 4 kb upstream sequences (4.2 kb EGP-2 promoter fragment) was identified and isolated from the BAC vector and cloned into the *Sac*II/*Bam*HI sites of the pBluescript SK plasmid (Stratagene, Inc., San Diego, CA). This construct was then subjected to further restriction mapping and DNA sequence analysis.

DNA sequence analysis.

DNA sequencing was performed using the Thermo Sequenase cycle sequencing kit (Amersham-Pharmacia, biotech.) with Cy5 labeled primers (Eurogentec) on the ALF-express sequencer (Amersham Pharmacia, biotech.). DNA sequence data were managed and analyzed by the DNA Star computer program (DNA Star Inc., USA). Consensus sequences of transcription factor binding sites were identified using MacVector and by searching the TRANSFAC v3.2 database using Transcription Element Search Software (TESS, . Pairwise sequence alignments were performed using the FASTA programs ALIGN and LALIGN.

GFP and Luciferase plasmid construction and assays

The 4.2 kb EGP-2 promoter fragment was digested with *Xma*III and subcloned into the *Not*I site of the pBluescript KS vector (Stratagene, Inc., San Diego, CA) generating two different constructs containing the insert in both orientations. By digestion with *Sac*I/*Xho*I the fragment was cloned from one pBluescript construct into the GFP reporter plasmid pEGFP-1 (Clonetech, Palo Alto, CA, USA), while by digestion with *Xho*I/*Sac*II from the other pBluescript construct the same EGP-2 promoter sequence was cloned into the luciferase reporter pGL3 enhancer vector (Promega Inc., Madison, WI, USA). The 3.6 kb promoter sequence cloned into these reporter vectors starts from –83 to –3508 containing the transcription start site and putative binding sites for Sp1 and AP-1 in the 5' untranslated region of exon 1 but not the ATG. Deletion constructs of the 3.6 kb EGP2 promoter region containing pEGFP-1 vector further referred to as $p39^{E}$, were generated using the *double-stranded* Nested Deletion Kit form Pharmacia (Amersham-Pharmacia, Biotech.). 22

constructs were generated following the manufacturers protocol using *Bgl*II to generate the recessed 3'-ends which were filled in with thionucleotides to make them nuclease resistant and *Spe*I to create a 5'-overhanging nuclease-sensitive end. The generated constructs chosen to be used in transfection experiments were; $p39^{E}$ (-3508/-83); $P39^{E4-7}$ (-2898/-83); $P39^{E17-1}$ (-2411/-83); $P39^{E15-2}$ (-2168/-83); $P39^{E7-2}$ (-1211/-83); $P39^{E9-2}$; $P39^{E4-1}$ (-871/-83); $P39^{E7-3}$; $P39^{E11-1}$ (-533/-83); $P39^{E12-3}$ (-238/-83), and $P39^{E12-2}$ (-170/-83). The numbers between the brackets refer to the positions towards the ATG in the GA733-2 genomic clone. GFP expression was studied both by microscopic and flow cytometric analysis using the Leica Quantimed 600 (Leica, Rijswijk, The Netherlands) and the Coulter Elite Cytometer (Coulter Electronics, Hilaleah, FL, USA). Luciferase activity was measured using the Promega luciferase assay system (Promega Inc., Madison, WI, USA) and light output recorded by the Anthos, Lucy 1 luminometer (Anthos labtec instruments, Salzburg, Austria).

EGP-2-EGP-2 and EGP-2-CD constructs

The –83 to –3508 EGP-2 promoter region was cloned upstream of the EGP-2 cDNA by exchanging the luciferase gene of the pGL3 vector for the EGP-2 cDNA in which the EGP-2 promoter had already been cloned as described above. Furthermore this EGP-2 promoter region was cloned as a *SpeI/NheI* fragment upstream of the E. coli Cytosin Deaminase DNA, situated in *NheI/Pme*I sites of the pcDNA 3.1(+) (Invitrogen).

Generation of EGP-2 transgenic mice.

A 55 kb *Xho*I DNA fragment containing the human EGP-2 gene was isolated from a BAC clone (Genomesystems Inc, St. Louis, Missouri). This genomic BAC clone was identified using 920 bps of the 5' region of the human EGP-2 cDNA derived from the GA21726-22R vector, kindly provided by Dr. Linnenbach (Wistar Institute, Philadelphia, USA).

The presence of GA733-2 genomic sequences was determined by PCR of exon 2-3, sense strand, 5'-ATAATAATCGTCAATGCCAGTGTA, antisense strand 5'-ATCATAAAGCCCATCATTGTTCT and exon 9 (sense strand 5'-TCAGATAAAGGAGATGGGTGAGA, antisense strand 5'-GGCAGCTTTCAATCACAAATCAG. Restriction analysis and subsequent Southern blotting using the upstream SacII/SalI fragment or the 1.5 kb EGP-2 cDNA as probe

it was set that at least 10 kb upstream and 4 kb downstream regulatory sequences were present. The 55 kb DNA fragment was introduced into oocytes of FVB/N mice according to standard methods . Three mice were found positive by PCR and Southern blot analysis for the *EGP-2* transgene. Of these founders, two lines transmitted the transgene to their progeny. Both lines were fertile and healthy and expressed the EGP-2 protein and one line was selected for further studies. For investigation of tumor growth and humoral immunity the EGP-2 transgenic FVB/N mice were crossed with C57/Bl6 wild type mice.

Immunohistochemical analysis.

Tissue-culture supernatant of the hybridoma MOC31 (anti-EGP-2; IgG1) was purified by protein A column chromatography (Pharmacia, Uppsala, Sweden) and biotinylated . Immunoperoxidase stainings were performed on 5-µm-thick, air-dried cryosections made from snap frozen biopsies. After acetone fixation and rehydration, antibody was applied and incubated at room temperature for 1 h.

Cell lines.

The murine B16.F10 melanoma (ATCC) and the EGP-2 transfected B16.F10 melanoma, B16.B16.C215 kindly provided by Dr. Dohlsten , were maintained in DMEM (Gibco BRL, NY, USA) supplemented with 50 µg/ml gentamycin sulfate (Biowhittaker, Vervier, Belgium); 2 mM L-glutamine (Gibco BRL); 10% FCS (Bodinco, Alkmaar, The Netherlands) at 37°C in humidified 5% CO_2 atmosphere. The human EGP-2 expressing rectum adenocarcinoma cell line SW948 (ATCC) was cultured as above and used to score antibodies in the serum of mice.

Tumor induction.

Subcutaneous tumors were induced by s.c. injection of $5x10^5$ B16.F10 or B16.B16.C215 cells in the right or left flank of trangenic mice or non-transgenic controls. Tumor development was determined by palpation at 14 to 21 days after induction.

Antibodies

Undiluted tissue-culture supernatant of the anti-EGP-2 hybridoma MOC31 (IgG1) was used to identify the presence of EGP-2 both immunohistochemically as well as on Western blot. An anti-bacterial cytosine deaminase antibody kindly provided by Dr. Haack, Heidelberg, Germany was used to identify CD positive cells immunohistochemically in a 1:50 dilution. The anti-GFP polyclonal antibody was obtained from Molecular Probes, Eugene, OR, USA and diluted 1:200. Horseradish-peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig (Dakopatts, Glostrup, Denmark) were used to detect the specifically bound antibodies.

SDS-PAGE and Western-blotting.

Detection of the human EGP-2 protein was performed on cell lysates. The cells were homogenized in 250µl, 250 mM Tris-HCL (pH 7.8), after which protein was further extracted by 5-times freeze /thaw cycling. Protein concentration was determined by the method of Bradford (41) (Bio-Rad Laboratories) on serial dilutions of the lysates. 12µg protein was mixed with an equal volume 2× SDS-PAGE sample buffer (42) without 2-mercapto-ethanol and heated at 100°C for 5 min. SDS-PAGE (42) was performed using the BioRad mini-protean II system with 10% polyacrylamide gel. Samples were semi-dry electroblotted onto nitrocellulose filters (Amersham, Chalfont, UK). After blotting, the filters were blocked overnight with 5% nonfat dried milk in PBS supplemented with 0.1% Tween-20. Filters were incubated with MOC31 hybridoma supernatant, washed, after which specific binding of the antibody was visualized using the ECL detection kit of Amersham, Chalfont, UK.

Immunohistochemistry

Cytospin slides of acetone-fixed cells were evaluated for EGP-2, CD, or, GFP expression by incubating with the relevant antibody at RT for 1 h. Peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit Ig, diluted 1:50 in PBS containing 1% normal serum, in combination with 0.01% H2O2 and AEC (Sigma, Bornhem, Belgium) as substrates, were used for specific staining. Counter staining was performed using a Mayers hematoxylin solution (Merck).

Results

We isolated a BAC clone containing the 14 kb GA733-2 genomic sequences including its own 10 kb upstream and at least 30 kb downstream regulatory sequences as determined by Southern blot analysis and PCR. Screening of a BAC genomic library with a 5' GA733-2 probe yielded one positive clone, which was characterized by restriction enzyme mapping, PCR-screening, and nucleotide sequencing. To define the GA733-2 promoter region, restriction analysis with *Sac*II and double digests with *Sac*II and a number of other enzymes were carried out. Since a *Sac*II site is positioned ~ 40 bp downstream of the ATG it was determined by Southern blot hybridization with the 5' GA733-2 probe that the BAC clone contained at least 10 kb of GA733-2 upstream sequences (*Sac*II digestion alone). Digestions with *Sac*II and *Eco*RV or *Sac*II and *Bgl*II revealed bands containing exon1 and aproximately 5 or 4 kb of upstream sequences, respectively (*Eco*RV, *Bgl*II digestion). The *Sac*II/*Bgl*II fragment was isolated, cloned, and further analyzed.

Sequencing of the cloned *Sac*II/*Bgl*II 5' GA733-2 promoter region in both directions yielded ~ 4 kb of sequence upstream of the longest reported 5' untranslated region of the EGP-2 cDNA (Salza) (Figure 2). This upstream region lacked canonical TATA and CAAT boxes commonly found within 100 bp upstream of the putative transcription sites. The 5'-upstream sequences did contain several putative *cis*-acting regulatory elements.

The complete nucleotide sequence of the approx 4.2kb *Bgl*II - *Sac*II fragment was determined. Exonuclease (Nested Deletion Kit from Pharmacia) was used to generate deletions from the 5' end of the promoter. The deleted promoter clones were also sequenced. The names of the generated deletions are mentioned to the right of the figure 1: $p39^{E4-7}$, $p39^{E17-1}$, $p39^{E15-2}$, $p39^{E7-2}$, $p39^{E4-1}$, $p39^{E11-1}$, $p39^{E12-2}$ and $p39^{E12-3}$. $p39^{E}$ was derived by cloning the approx 3.6kb *Xma*III restiction fragment. Putative transcription factor binding sites are marked (Sp-1, Ap-1, Ets) in figure 1, of these Ets is a known epitheliumspecific transcription factor (B.Wasylyk, S.L.Halm and A.Giovane (1993)The Ets family of transcription factors. Eur.J.Biochem. 211:7-18). In addition, Sp-1 in combination with a Ets binding site in close proximity is known to regulate epethelium specific gene expression (J.H.Lee, S.J.Jang, J.M.Yang,

N.G.Markova and P.M.Steinert (1996) The proximal promoter of the human transglutaminase 3 gene, J.Biol.Chem. 271:4561-4568). The previously published sequence by Linnenbach et al (Mol.Cell.Biol. (1993)13,1507-15, genbank accession M93029) is boxed at the bottom of the figure. Of this sequence the cDNA is given in italics, whilst the protein encoding sequence (starting with ATG) is given in bold italics. The putative transcription start site, as was suggested by Linnenbach et al is marked with a hooked arrow.

The epithelial glycoprotein 2 (EGP-2), also known as Ep-CAM or the pancarcinoma associated protein 17-1A, encoded by the GA733-2 gene, is expressed as a stable transmembrane protein at high levels on the surface of most carcinomas. Despite the fact that EGP-2 is also expressed on normal simple epithelial tissue, EGP-2 is regarded as an attractive target for anti-cancer immunotherapeutical treatment strategies. To explore the mechanisms regulating the expression of the EGP-2 gene, 3.6 kb of sequences upstream from the transcription start site were assessed for their ability to control the expression of the EGP-2 cDNA, the green fluorescent protein (GFP), and the luciferase reporter genes. Analyses of the expression of these constructs in transiently transfected EGP-2 positive and EGP-2 negative carcinoma and non-carcinoma derived cell lines revealed epithelial specific expression. Deletion analyses defined a basic proximal promoter region, which for example confers epithelial-specific expression to the GFP reporter gene. Using these sequences to direct the prodrug 5'CU converting enzyme cytosine deaminase it was possible to discriminate between EGP-2 expressing and non-expressing cells by the cytotoxic effect of the drug. As these EGP-2 sequences confer promoter/enhancer activity to reporter genes in a tissue specific manner, they are useful for gene therapy in EGP-2 overexpressing carcinomas.

By injecting a 55 kb GA733-2 spanning genomic DNA fragment isolated from this BAC clone into FVB/N mice oocytes, fully immunocompetent mice transgenic for the human EGP-2 protein were generated. Expression of the human EGP-2 protein in the generated transgenic mice was confined to the lung, kidney, pancreas, stomach, colon, small intestine, gonads and not to the hart, muscle, brain, spleen, and liver tissue in two EGP-2 transgenic mice lines as determined by RT-PCR and Western blotting. Immunohistochemical analysis revealed that the EGP-2 promoter sequences directed the EGP-2 expression to the membrane of corresponding epithelial cells

revealing a distribution pattern similar to the human situation. In the kidney strong EGP-2 expression was observed in the epithelial cells of the Henle's loop whereas the Bowman's capsule and the proximal and distal tubuli stained weakly positive . The stratified bronchal epithelium of the major airway, the alveolar epithelium and epithelial tissue found in the mucus glands of the broncheal mucosa stained also positive for EGP-2 expression . Of the gastointestinal tract the villus and crypt epithelium of small intestine and colon as well as the gastric surface epithelium of the stomach demonstrated EGP-2 expression whereas the gastric glands appeared to be negative. EGP-2 expression was also observed in the glandular epithelium of the endometrium, the tubili seminiferi of the testis and in the valopian duct epithelium of the ovary. Furthermore, the exocrine and ductular epithelium of the pancreatic tissue stained positive for EGP-2, whereas the endocrine epithelial stained only weakly positive. In the liver EGP-2 expression was observed in the bile duct epithelium, whereas the hepatocytes were negative and in the thymus epithelia with especially the Hassall's corpuscles stained positive for EGP-2. No EGP-2 expression was observed in hart, muscle, spleen and T-, B, and dendritic cells nor was EGP-2 shed in the blood as determined by inmmunohistochemical and FACS analysis. This observed transgene expression pattern was integration site-independent but copy number dependent as established by FISH analysis (results not shown).

The human EGP-2 protein consists of an extracellular domain with two EGF-like repeat motifs, a transmembrane region of 23 hydrophobic amino acid residues, and a relatively short 26-residue highly charged cytoplasmic domain with an internalization motif. Upon transfection with EGP-2, cells incapable of intercellular adhesion formed aggregates suggesting a homotypic adhesion function for EGP-2. Several other experiments pointed to a role of EGP-2 as signaling molecule leading to a regulation of proliferation and differentiation of epithelial cells and also a morphoregulatory role was credited to the EGP-2 protein. However, the exact role of EGP-2 in epithelial cell functioning remains to be elucidated. The high-affinity mAb MOC31 recognizes an epitope in the first EGF-like repeat of the extracellular domain of the EGP-2 molecule. Specific MOC31 mAb binding to EGP-2 expressed on the membrane of the normal epithelial tissues of the EGP-2 transgenic mice and comparison of this staining pattern with the MOC31 staining pattern of human EGP-2 expressing normal epithelial tissues demonstrated an accurate expression of the

transmembrane glycoprotein in this EGP-2 transgenic mouse model. However, though being defined as a homotypic adhesion molecule, no evidence of adhesion was observed in the EGP-2 transgenic mice. Survival was identical in EGP-2 transgenic mice and wild-type mice as analyzed for 12 months, despite expression of EGP-2. Additionally, strong expression of EGP-2 on the ovary duct and sertoli cells did not influence fertility of the transgenic animals. Transgenic female animals gave normal birth to viable transgenic offspring. These observations debate the function of EGP-2 as a homophilic adhesion molecule. This function, however, was established in cells lacking their own means of cell-cell interactions and not in the presence of mEGP-2. The presence of mEGP-2 can also explain why other functions ascribed to EGP-2 are not observed, like active proliferation, whether normal or neoplastic. No neoplastic lesions or morphological aberrations were observed in the EGP-2 transgenic mice tissues analyzed, suggesting a bystander role of the EGP-2 protein in these processes. However, in the mammary gland of the MMTV-EGP-2 transgenic mice ductal hyperplasia was observed and differentiation of lobular and ductal cells was affected by the ectopically expressed human EGP-2. The observed differences between these EGP-2 transgenic mice models might be explained by the differences in 5' regulatory sequences or intron specific regulatory elements used as has been described for several transgenically expressed genes and promoters .

Since EGP-2 is one of the best-studied tumor-associated antigens frequently used as a target for experimental and clinical cancer immunotherapy, we wanted to induce human EGP-2 positive cancer in our transgenic mice. Previously the B16.F10 murine melanoma cell line has been stably transfected with the GA733-2 cDNA and was called B16.C215. This cell line was then used to study the role of antibody-targeted super antigens in immunotherapy in animal models mimicking human malignant conditions (Dohlsten 1995). To adept this C57/Bl6 model on the current EGP-2 transgenic FVB/N mice, transgenic FVB/N/C57/Bl6 hybrid mice were generated and tumor growth was monitored after subcutaneous induction of EGP-2 positive B16.C215 or EGP-2 negative B16.F10 tumors. The EGP-2 expression pattern remained the same in the hybrid genetic background as was established by immunohistochemical analysis (results not shown). No significant difference in growth of the s.c. induced B16.F10 or B16.C215 tumors was observed between EGP-2

transgenic mice or wild-type littermates. However, approximately 60% of the transgenic animals demonstrated intraperitoneal growth of the B16.C215 tumor upon s.c. induction. This intraperitoneal invasive growth was not observed in nontransgenic animals or in transgenic animals upon s.c. induction of a B16.F10 tumor in 3 independent experiments with 4 animals per condition. Tumor growth in the peritoneal cavity surrounded but never invaded the organs present and was associated with an increased lethality among the transgenic animals s.c. injected with B16.C215 tumor cells (3b). Another striking difference between transgenic and nontransgenic animals upon tumor induction was the observed spleen enlargement in wild-type animals. This spleen enlargement could only be observed in transgenic animals in relation to intraperitoneal growth. To investigate the meaning of this observed spleen enlargement blood serum of all animals was tested on anti-EGP-2 reactivity using constitutively EGP-2 expressing human cells and as a control EGP-2 negative human cells. An anti-EGP-2 humoral immune response was observed in the non-transgenic mice whereas no such response could be observed in the serum of the EGP-2 transgenic mice. However, no spleen enlargement was observed in the transgenic animals upon induction with B16.F10 cells as well demonstrating a complete tolerance, irrespective of the transgene, which is not associated with enhanced tumor growth. This is interestingly since both the B16 melanoma cells and the EGP-2 protein, are considered poorly immunogenic. B16 mouse melanoma cells are poorly immunogenic due to expression of only minute amounts of MHC class I molecules (Dohlsten, 1995), whereas the high degree of EGP-2 with its murine homologue is responsible for its poor immunogenicity in mice. Also in humans EGP-2 is found to be poorly immunogenic. About 15% of colorectal carcinoma patients had IgG autoantibodies against EGP-2, while no healthy donors examined did. Although the frequency was higher with more advanced clinical stage, no significant association between the presence of auto-EGP-2 antibodies and survival was noted.

This seemingly paradigm between EGP-2 expressed by the transgenic animal demonstrating no relation between EGP-2 expression and proliferation or neoplasia and the EGP-2 expressing B16.C215 tumor cells which demonstrate enhanced invasive properties in comparison to its parental B16.F10 cells in EGP-2 transgenic mice was observed previously in several experiments. Using both human tissue

culture cells and animal models it was established that (over-) expression of EGP-2 correlated with both benign and malignant proliferation of epithelial cells . The EGP-2-transgenic mouse tumor-model presented here seems to be an excellent tool to study this dualistic role of EGP-2 in tumor development and the additional signals responsible for either phenotype. Specifically when these EGP-2 transgenic mice are cross-bred with mice that are genetically predisposed to develop different types of tumors . In addition, since the endogenous EGP-2 regulatory sequences have been used to direct EGP-2 expression in these transgenic mice they can also be used to evaluate the importance of EGP-2 during embryonic development or morphogenesis of individual tissue. Though relatively little information is available concerning the expression of the EGP-2 gene during human embryonic development, several studies suggest an important role for EGP-2 during embryogenesis. EGP-2 is expressed by the majority of human epithelial neoplasias, and as such has been a target for immuno- and gene therapy strategies. Anti-cancer strategies targeting the EGP-2 antigen require an appropriate pre-clinical model to study the efficacy and toxicity of these strategies in order that in the near future strategies targeting this molecule can be applied safely in clinical trials to combat carcinomas in patients. The EGP-2-transgenic mouse tumor-model provided here is an excellent tool to study these new therapeutic strategies. Not only does it express the human EGP-2 protein accurately and with a distribution pattern similar to the pattern seen in humans (Table 1), but it displays also the immunological tolerance frequently observed in cancer patients against tumor antigens. This is of great significance since the mechanisms that regulate immunological tolerance to tumor antigens are formidable obstacles that withstand effective tumor immunotherapy in cancer patients.

Table 1 Epithelal glycoprotein-2 (EGP-2) distribution in EGP-2 transgenic FVB/N mice

Tissue	Transgenic mice			Nontransgenic mice		
	$MOC31^{bio}$	UBS 54	PBS	$MOC31^{bio}$	UBS 54	PBS
Liver (Bile duct)	+	+	–	–	–	–
Liver (Hepatocytes)	–	–	–	–	–	–
Pancreas	+	+	–	–	–	–
Small intestine	+	+	–	–	–	–
Colon	+	+	–	–	–	–
Lung	+	+	–	–	–	–
Kidney	+	+	–	–	–	–
Stomach	+	+	–	–	–	–
Gonads	+	+	–	–	–	–
Thymus(Hassall's corpuscles)	+	+	–	–	–	–
Brain	–	–	–	–	–	–
Heart	–	–	–	–	–	–
Skin	–	–	–	–	–	–
Spleen	–	–	–	–	–	–
Muscle	–	–	–	–	–	–

Figure legends

Figure 1

EGP-2 promoter analysis

Nucleotide sequence of the approx 4.2kb *Bgl*II - *Sac*II fragment was determined. The names of the genereted deletions are mentioned to the right of the figure: $p39^{E4-7}$, $p39^{E17-1}$, $p39^{E15-2}$, $p39^{E7-2}$, $p39^{E4-1}$, $p39^{E11-1}$, $p39^{E12-2}$ and $p39^{E12-3}$. $p39^{E}$ was derived by cloning the approx 3.6kb *Xma*III restiction fragment. The end of each deletion is marked with "[". Putative transcriptionfactor binding sites are marked Sp-1, Ap-1, Ets. The putative transcription startsite is marked with a hooked arrow. Sizemarkers to the left of the figure are relative to this putative transcription start site.

Figure 2. EGP-2 promoter analysis. Deletion mutants of the EGP-2 promoter were fused to the EGFP (enhanced greeen fluorescent protein) and transfected into non-epithelial cells, ie FLF (human fetal lung fibroblasts) and HUVEC (human umbilical vein endothelial cells) or epithelial cells SW948 (human colorectal carcinoma) or as a transfection and expression control into COS-7 cells (immortalised kidney epithelial cells derived from the African Green Monkey). Construct names (of Figure 1) are given above the line that represents the promoter. The numbers indicate the distance from the putative transcription start site that was given by Linnnenbach et al (1993). In the figure this transcription start site is denoted as 1.

Constructs $p39^{E12-2}$ and $p39^{E12-3}$ gave virtually no transcription in all cells types that were tested, while $p39^{E11-1}$ only gave a marginal expression in all lines tested. Thus $p39^{E11-1}$ contains the basic minimal promoter that can bind the RNA polymeraseII complex. CoMplete epitheliumspecific expression was found upon transfection with fusion constructs containing at least 778bp upstream of the putative transcription start site ie $p39^{E4-1}$ comprises binding sites for epitheliumspecific transcription factors.

Similarly, constructs containing the promoter sequences from p39E (approx 3.4 kb upstream) fused to the luciferase gene, the EPG-2 cDNA sequence, the Cytosine Deaminase (CD) gene showed epitheliumspecific expression in the same cell types as mentioned above.

Claims

1. An isolated and/or recombinant nucleic acid comprising a tissue specific promoter or functional fragment thereof allowing for expression of a nucleic acid of interest operably linked to said promoter or functional fragment thereof in a cancer cell wherein said expression in said cancer cell is essentially carcinoma selective.

2. A nucleic acid according to claim 1 wherein said carcinoma comprises lung carcinoma.

3. A nucleic acid according to claim 1 or 2 comprising a nucleic acid or functional fragment thereof as shown from about position -778 to about position -422 in figure 1 or a nucleic acid functionally equivalent thereto.

4. A nucleic acid according to claim 3 derived from a human.

5. A nucleic acid according to anyone of claims 1 to 4 further comprising a nucleic acid of interest.

6. A nucleic acid according to anyone of claims 1 to 5 further comprising an inducable or suppressible promoter or functional fragment thereof.

7. A nucleic acid according to anyone of claims 1 to 6 further comprising a suicide gene or functional fragment thereof.

8. A vector comprising a nucleic acid according to anyone of claims 1 to 7.

9. A gene delivery vehicle comprising a nucleic acid according to anyone of claims 1 to 7.

10. A host cell comprising a nucleic acid according to anyone of claims 1 to 7, a vector according to claim 8 or a gene delivery vehicle according to claim 9.

11. An experimental animal comprising a cell according to claim 10.

12. Use of a nucleic acid according to anyone of claims 1 to 7, a vector according to claim 8 or a gene delivery vehicle according to claim 9 for the preparation of a medicament.

13. Use according to claim 12 wherein said medicament is for the treatment of cancer.

14. A medicament comprising a nucleic acid according to anyone of claims 1 to 7, a vector according to claim 8 or a gene delivery vehicle according to claim 9.

15. A method for the treatment of cancer comprising administering to a patient a nucleic acid according to anyone of claims 1 to 7, a vector according to claim 8 or a gene delivery vehicle according to claim 9.

16. A method for evaluating a possible treatment of disease comprising testing such treatment on a host cell according to claim 10 or an animal according to claim 11.

17. A method according to claim 16 wherein said treatment comprises treatment of disease comprising non-squamous epithelium.

18. A method according to claim 16 or 17 wherein said disease comprises carcinogenesis.

Fig. 1

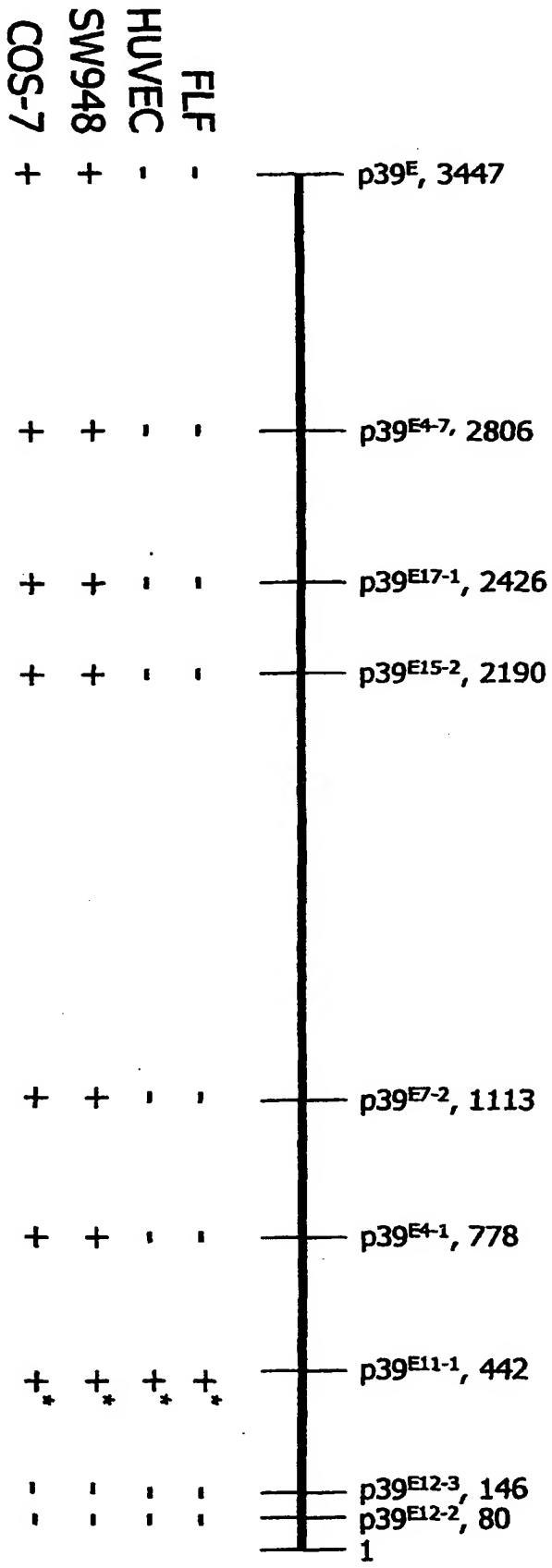
EGP-2 promoter sequences

BglII
-3967.AGATCTAGAA TAGAGAGGGA TTTGCTGCAT AGTGGTTAAG GACTTTTACT CTTCATTCTA TATAAAGGAC TTTTGTTTTC
-3887.TACTCATCTA TTACTTATGG GATAACAAAA ATTTTTAGAA CTGGTAGTCT AATTTTATAT ATATATATAT ATATATATAT
-3807.ATATATATAT ATATATATAT ATATATTTTT TTTTTTTTTT TTTTAGACAG AGTTTTGCTC TTGTTGCCCA GGCTGGAGTG
-3727.CAATGGCATG ATCTTCGCTC ACCACAACCT CCGCCTCCTG GGTTCAAGTG ATTCTCCTGC CTCAGCCTCC CAAGTATCTG
-3647.GAATTACAGG CATGTGCCAC CATGCCCAGC TAATTTTTAT ATTTTTAGTA GAGACAGGTT TTCACCAGGT TGCCCAGGCT
-3567.GCTCTCAAAC TCCTGACCTC AAGTGATCCA CCCGCTTTGG CCTCCCAAAG TGCTGGGATT ACAGGCGTGA GCCACCATGC
-3487.CTAGCCTGAA AATATTAATA AATGTGCTTA AATATGGCAC TAGAACTACA AAAGATTCAC.AATTAAAACA TAAAACGAGT
XmaIII
-3407.AATTTTGAGC AAAGAATGAC AAATTGAGAA GGTGTTAATG AGGTACTAAA ATAAACAATA CCGGCCG[GTGCAGTGGCTCA p39[5]
Sp-1
-3327.TGCCTGTAAT CCCAGCACTT TGGGAAGCTG AGGCGGGTGG ATCACCTGAG GTCAGGAGTT CAAGACCAGC CTGGCCAACG
-3247.TAGTGAAACC CGGTCTCTAC TAAAAATACA AAAATTAGCC GGGCGAGGTG GCAGGCGCCT.GTAATCACAG CTACTCGGGA
-3167.GGCTGAGACA GGAGAATTGC TTGAACCCAG GAGGTGGAGG TTGCAGTGAG CTGAGAACAC GCCATTGTAC TCCAGCCTGG
-3087.GTAACAAGAT TGAAACTCTA TCTTAAAAAA AAAAAAAAGG CGGACACGGT GGCTTGCACC TGTAATCCCA GCACTTTGGG
-3007.AGGCCGAGGC AAGAGGATCA CAAAGTCAGG AGATCAAGAC CATCCTGGCC AACATGGTGA AACTCTGTCT CAACTGAAAA
-2927.TACAAAAATT AGCCGGGTGT GGTGGTGGGC GCCTGTAATC CCAGCTATTC AGGAGGCTGA GGCAGGAGAA TTGCTTGAAC
-2847.CCAAGAGGTG GAGGTTGCAG TCCGCCAAGA TCATGCCACT GCACTGCAGC TTGGGTGACA GAGCAAGACC CCATCTCAAA
-2767.AAAAAAAAAA AAGAAAAAAT ACCCTGGATC AGCCGGGTGT GGTGGCTCAA GCCTGTAATC CC[AGCACTTTGGGAGGCTGA p39[E4-7]
-2687.GGTGGGCAGA TCACCTGAGG TCAGGAGTTC AAGACCAGCC TGACCAACAT GGAGAAACCC CATCTCTACT AAAAATACAA
-2607.AAAATTAGCC GGACGTGGTG GCACATGCTT GTAATCCCAG CTACTCAGGA GGCTGAGGCA GGAGAATTGC CTGAATCCGG
-2547.GAGGCGGAGG TTGTGGTGAG GTGAGATGAT GCCATTGCAC TCCAGCCTGG GCAACAAGAG CAAAACTCTG CCTCAAAAAA
AP-1
-2447.AGAAAGAAAA AAAAAAAAGA AAGAAAAGAA AAAATACCCT GGATGTATAC TCAGATACAA TGAGTCAGAG ATTAGTCTGG
-2367.TATTTTGTCA TTTATTTAAT AATTATGCTT ACTCAATTCA CTT[TATTGTAATTAACAATA AATAGCTGTC CAGTTATAAG p39[E17-1]
Ets
-2287.AAGATGAAGT TCTCCCGATT AGGTAAACAG ATTTAGACCT CAGAATGGAA CATTTTGCCA ATAAAGCCAC AATAACCAGT
-2207.TAGTTTATTC TTGGGAAAAG TATATGTAAT TTGGAGAAAG GCAAACTTCC TGAAAACATC CAAAATTCAG CAGACAACAA
Ets
-2127.AAATCTGGTT AACTTGTTCC TGATTTGTTA GTACTATTC[TTTTTTTTTTG TTTGTTTGTT TTTTTTTTTT GAGACGGAGT p39[E15-2]
-2047.TTCGCTCTTG TTGCCCAGGC TGGAGTGCAA TGGCGAAATG TTGGTTCACT GCAACCTCTG CCTCCCAGGT TCAAGTGATT
Sp-1
-1967.CTCCTGCCTC AGTCTCCTGA GTAGCTGGGA TTACAGGCGC CCGCCACCAC GCCTGGCTAA CTTCTTGTAT TTTTAGTAGA
Sp-1
-1887.GACGGGGTTT CACCATGTTG GCCAGGCTGG TCTCGAACTC CTGACCTTAG GTGATCCGCC CGCCTCGGCC TCCCAAAGTG
-1807.CTGAGATTAC AGGCATGAGC CACCGTACCT GGCCTAAATA CCTTATTTCA TATACCACGT GAAATTTAAA TTATACAAAA

Fig. 1, contd.

-1727.CAAATTATAG AGGTACTTAG AACAGCATGA CTATTTACAT TAATCAACTT GCCGGCACTT CAACAGAATA CAACATAGAA
-1647.ATGATTGTTT TAATATAAAC ATAAGCTTTG ATTTGACATA TACTTGTAGA AATTAATCAA ACTTAGCTGA ATCTTAAAAT
-1567.TGCTTTTTTA CCTTTTCCTCT TTTTTTTTTT TTTTTTTATT TTTTGAGATG GAGTCTTGCT CTGTTGCCAG ACTGGAGTGC
-1487.AGCGGTTTGG TCTCGGCTCA CCGCAACCTC CGACTCTCTG GTTCAAGCGA TTCTCCTGCC TCAGCCTCCT GAGTAGCTGG
-1407.GATTACAGGT GCCTGCCACC ACACCTGGCT ACTTTTTGTA TTTTTAGTTG AGATGGGTTT CACCATGTTG GCCAGGATGG
-1327.TCTCGAACTC CTGACCTCGG ATCTGCCCCC AGCAAGGTGC TGGGATTACA AGCATGAGCC ACCGTGCCCA
-1247.GCCTCCTTTC CTCTTTTAA CTCTTACTTT TATGATTTCT TTAGTGGATA AAAAGCTTTT AAAAAATAGG TTACAATGAT
-1167.ATTACAGCTA ACAAAAAATA ACATTTAAAA ACACTAAATA GTATATATAT GAAGTATTTA TAATTATTTT AATATTGTAA
-1087.TAATATAGTG TGTTGTGATT TGAATTCATC TGCACGGAAA TCGATTACTG TCCTTTCTTT CTATTTCCCT ATAT[TTTCTT p39E7-2
-1017.TCCGAAGCGT CATCAACATT TTGGTTCTTT AATAGTAACC AAAACCCGAA ATCATCTCGG TTCTCAGTAT TTGGCTCTAT
-937.GGGAACACCT TTTCTTTTCT CTCTTTTTTT TTTTTTTTGA GACGGAGTCT TGCTCCTGTC GCCCAGGCTG GAGTGTAATG
-857.GCACGATCTC TGCTCACTGC AACCTCAGCC TCCCCAGTAG CTGGGATTAC AGGCATGCGC CACCACGCCC GGCTAATTTT
-777.GTATCTTTTA GTAGAGACGG CATTTCCTCA TGTTGGTCAG GCTGGTCTCG AACTTCAAAC CTCAGGTGAT CCGCCCGCCT
-697.CGGCCTCCC[AAAGTGCTAGG ATTACAGGCG TGAGCCACCG CGCTCAGCCT GGGAACACCT TTTCTTACAT CTTCAAGTGC p39E4-1
-617.TAGAAATGCT TATGAAAACG AAAAAAGAAT TATTAAGAGT AATTATAAAG AAACACTCAT TTTCTTCCCA AGAGAGCCAA
-537.GATTTCTCT TTCCTTTTCT TTCTTTTTTT TTTCTTTCTA ATTTCAAAGG AGTATAATTA AATTGCCAGG TAAAAGCTGA
-457.AAGGTTTTTT TTATAGTGTT CTGGAAGGTT CTCTGCCTGT GTTTGTATTT CCTTTAGCCT CCACGTTCCT CTATCCAGTT
-377.CCCGCACCCT TCCCCCCAGG CCCCATTCTT CAAGGCTTTC AGAGCAGCGC TCCTCGGTTA AAAGGAAGTC TCAGCACAGA p39E11-1
-297.ATCTTCAAAC CTCCTCGGAG GCCACCAAAG ATCCCTAACG CCGCCATGGA GACGAAGCAC CTCGGGCGGG GCGGAGCGGG
-217.GCGCGCGGGC CCACACCTGT GGAGAGGCCC GCGCCCCAAC TGCAGCGCCG GGGCTGGGGG AGGGGAGCCT ACTCACTCCC
-137.CCAACTCCCG GGCGCTGACT CATCAACGAG CACCAGCGGC CAGAGGTGAG CAGTCCCGGG AAGGGGCCGA GAGGGGGCGC
-57.[CGCCAGGTCGGGCAGGTGTG CGCTCCGCCC CGCCGCGCGC ACAGAGCGCT AGTCCTTCGG CGAGC[GAGCACCTTCGACGC p39E12-2,3
+23.GGTCCGGGGA CCCCCTCGTC GCTGTCCTCC CGACGCGGAC CCGCGTGCCC CAGGCCTCGC GCTGCCCGGC CGGCTCCTCG
+103.TGTCCCACTC CCGGCGCACG CCCTCCCGCG AGTCCCGGGC CCCTCCCGCG CCCCTCTTCT CGGCGCGCGC GCAGCATGGC
+183.GCCCCCCGCAG GTCCTCGCGT TCGGGCTTCT GCTTGCCGCG GCGACGGCGA CTTTTGCCGC AGCTCAGGAA GGTGAGGCGC
+263.GCATTGGAGC AGAGTTGTGG AGCTGGCTTG GGCTGGGGGG CA

Fig. 2


COS-7
SW948
HUVEC
FLF
p39E, 3447
p39E4-7, 2806
p39E17-1, 2426
p39E15-2, 2190
p39E7-2, 1113
p39E4-1, 778
p39E11-1, 442
p39E12-3, 146
p39E12-2, 80
1

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P52075PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 01/00166	28/02/2001	01/03/2000
Applicant		
RIJKSUNIVERSITEIT GRONINGEN		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00166

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 A61K48/00 C12N15/63 A01K67/027 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K A01K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 00013 A (UNIV CALIFORNIA) 8 January 1998 (1998-01-08) page 15, line 5 -page 17, line 31; claims 46-61	1,2,5, 7-18
X Y	WO 98 13507 A (MCGILL UNIVERSITY) 2 April 1998 (1998-04-02) page 1, line 30 -page 2, line 2 page 7, line 22 -page 8, line 8	1,2,5, 7-18 1-5,7-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2001	20/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE EMBL DATABASE 'Online! Accession no. AC079775, 13 September 2000 (2000-09-13) WATERSTON R.H. : "Homo sapiens chromosome 2 clone RP11-295P2, WORKING DRAFT SEQUENCE, 8 UNORDERED PIECES" XP002173788 abstract	3,4
A	LINNENBACH A.J. ET AL.: "Retroposition in a family of carcinoma-associated antigen genes" MOLECULAR AND CELLULAR BIOLOGY, vol. 13, March 1993 (1993-03), pages 1507-1515, XP000566641	3,4
Y	page 1511, column 1, line 11 -page 1513, column 1, line 11; figure 3	1-5,7-18
A	FORNARO M. ET AL.: "Cloning of the gene encoding Trop-2, a cell-surface glycoprotein expressed by human carcinomas" INTERNATIONAL JOURNAL OF CANCER, vol. 62, no. 5, 4 September 1995 (1995-09-04), pages 610-618, XP000925819	3,4
Y	figure 7	1-5,7-18
A	BALZAR M. ET AL.: "The biology of the 17-1A antigen (Ep-CAM)" JOURNAL OF MOLECULAR MEDICINE, vol. 77, no. 10, October 1999 (1999-10), pages 699-712, XP000925817 the whole document	1-18
A	SIEMIENIAKO B. ET AL.: "Nuclear proteins from Capan-2 cell line form specific complexes with the 17-1A antigen gene promoter" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 186, no. 3, 14 August 1992 (1992-08-14), pages 1353-1361, XP000929804 the whole document	1-18

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1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00166

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BIRREN B. ET AL.: "Homo sapiens chromosome 4, clone RP11-77N9 map 4, WORKING DRAFT SEQUENCE,13 unordered pieces" EMBL DATABASE ENTRY AC018614; ACCESSION NO. AC018614, 16 December 1999 (1999-12-16), XP002144497	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 01/00166

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

[redacted]tion on patent family members

[redacted]ternational Application No
[redacted]CT/NL 01/00166

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9800013 A	08-01-1998	AU 3583797 A	21-01-1998
		US 5976800 A	02-11-1999
WO 9813507 A	02-04-1998	AU 4292797 A	17-04-1998
		EP 0954590 A	10-11-1999

INTERNATIONAL SEARCH REPORT

ational Application No
PCT/NL 01/00166

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 A61K48/00 C12N15/63 A01K67/027 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K A01K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 00013 A (UNIV CALIFORNIA) 8 January 1998 (1998-01-08) page 15, line 5 -page 17, line 31; claims 46-61	1,2,5, 7-18

X	WO 98 13507 A (MCGILL UNIVERSITY) 2 April 1998 (1998-04-02)	1,2,5, 7-18
Y	page 1, line 30 -page 2, line 2 page 7, line 22 -page 8, line 8	1-5,7-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2001	20/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE EMBL DATABASE 'Online! Accession no. AC079775, 13 September 2000 (2000-09-13) WATERSTON R.H. : "Homo sapiens chromosome 2 clone RP11-295P2, WORKING DRAFT SEQUENCE, 8 UNORDERED PIECES" XP002173788 abstract	3,4

A	LINNENBACH A.J. ET AL.: "Retroposition in a family of carcinoma-associated antigen genes" MOLECULAR AND CELLULAR BIOLOGY, vol. 13, March 1993 (1993-03), pages 1507-1515, XP000566641	3,4
Y	page 1511, column 1, line 11 -page 1513, column 1, line 11; figure 3	1-5,7-18

A	FORNARO M. ET AL.: "Cloning of the gene encoding Trop-2, a cell-surface glycoprotein expressed by human carcinomas" INTERNATIONAL JOURNAL OF CANCER, vol. 62, no. 5, 4 September 1995 (1995-09-04), pages 610-618, XP000925819	3,4
Y	figure 7	1-5,7-18

A	BALZAR M. ET AL.: "The biology of the 17-1A antigen (Ep-CAM)" JOURNAL OF MOLECULAR MEDICINE, vol. 77, no. 10, October 1999 (1999-10), pages 699-712, XP000925817 the whole document	1-18

A	SIEMIENIAKO B. ET AL.: "Nuclear proteins from Capan-2 cell line form specific complexes with the 17-1A antigen gene promoter" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 186, no. 3, 14 August 1992 (1992-08-14), pages 1353-1361, XP000929804 the whole document	1-18

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00166

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIRREN B. ET AL.: "Homo sapiens chromosome 4, clone RP11-77N9 map 4, WORKING DRAFT SEQUENCE,13 unordered pieces" EMBL DATABASE ENTRY AC018614; ACCESSION NO. AC018614, 16 December 1999 (1999-12-16), XP002144497	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 01/00166

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9800013 A	08-01-1998	AU 3583797 A	21-01-1998
		US 5976800 A	02-11-1999
WO 9813507 A	02-04-1998	AU 4292797 A	17-04-1998
		EP 0954590 A	10-11-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P52075PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL 01/ 00166	28/02/2001	01/03/2000
International Patent Classification (IPC) or national classification and IPC	C12N15/85	
Applicant	RIJKSUNIVERSITEIT GRONINGEN & Al.	

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 2 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consists of a total of _____ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/10/2001	17/04/2002
Name and mailing address of the IPEA/ European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer DE BUNDEL E R J Tel. (+49-89) 2399 2828

EUROPÄISCHES PATENTAMT - EUROPEAN PATENT OFFICE - OFFICE EUROPÉEN DES BREVETS

I. Basis of the report

The basis of this international preliminary examination is the application as originally filed.

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The question of whether the claimed invention appears to be novel, to involve an inventive step, or to be industrially applicable has not been the subject of the international preliminary examination in respect of the claims which have not been searched (Article 17(2)(a) or (3) and Rule 66.1(e) PCT); see also international search report).

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability

To the extent that the international preliminary examination has been carried out (see item III above), the following is pointed out:

In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims, which have been the subject of an international search report, does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claim references).